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FURTHER EXPERIMENTS ON ARTIFICIAL PARTHENOGENESIS IN STARFISH EGGS, WITH A REVIEW

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THE special physiological effect of fertilization or artificial activation in the resting egg cells of animals or plants is to initiate cell division, with the associated development, which may then proceed automatically to an advanced stage. It should be remembered, however, that effects of this general type are by no means confined to egg cells. The initiation of cell multiplication, growth, and differentiation in previously quiescent cells or tissues, as a result of some physical or chemical influence reaching them from their environment, is a widespread, if not universal, phenomenon in living organisms, illustrated more particularly in the various forms of response to developmental or morphogenetic stimuli. Every ontogeny, considered in its character as an integrated sequence of formative processes, depends on responses of this type. The inductive or organizing influence exercised by one region of the growing embryo on other regions includes such responses; and it is now known that the differentiation of the neural plate in the amphibian embryo may be initiated not only by the living cells of the dorsal lip of the blastopore but also by a variety of chemical and physical "stimuli" of a nonspecific kind. The morphogenetic stimulus may be of an "organizing" kind, or it may be a simple nonspecific initiatory or activating influence.[1] Artificial parthenogenesis, as a type of physiological process, falls in the latter category. The resting cell is aroused from a state of inactivity and proceeds to express its "latent" formative or developmental potentialities. Regenerative development and related phenomena, such as the production of supernumerary limbs in embryos as a result of trauma, may be regarded from a similar point of view (cf. Child, 1940, p. 38).

In higher animals definite chemical compounds, the hormones, acting singly or in combination, control the development, as well as the activity, of many special organs, especially those belonging to the reproductive system. The gonadotropic hormones of mammals and birds determine the special features of growth and differentiation, not only in the gonads themselves but also in accessory organs like the mammary gland and uterus, and the secondary sexual characters. In Amphibia the anterior pituitary hor-

[1] For a discussion of this difference cf. the recent book of Paul Weiss, *Principles of Development*, Part III (1939); see esp. pp. 366 ff.

mone initiates cell division and differentiation in the early oögonia (Rugh, 1937), even though the development of these cells past the oöcyte stage requires fertilization or some other form of activation. The initiation of cell division is not the only phenomenon to be considered in activation processes of this type. Enlargement and differentiation of cells may occur independently of cell division; even in unfertilized egg cells differentiation may occur without cell division under certain activating treatments, as in the *Chaetopterus* egg (F. R. Lillie, 1902). The arrest of growth and development at a certain stage and their reinitiation by later physiological changes of condition are not infrequent in organisms, as illustrated, for example, in the dormant stages which occur during the metamorphosis of many animals. In the diapause of insects growth ceases temporarily, and respiration falls to a low level; both may afterward be renewed either under normal environmental conditions or as a result of certain kinds of artificial treatment (cf. Bodine, 1932; Bodine and Boell, 1934). The cells whose development is thus arrested have been called "blocked cells"; in a somewhat similar sense (as in certain characteristic features of their metabolism, such as insensitivity to cyanide) unfertilized egg cells (e.g., of Arbacia) correspond to blocked cells of a certain kind.

Analogous phenomena occur in plants, where, also, artificial parthenogenesis is well known (for a recent study cf. Goddard, 1935). Lately the growth-promoting hormones of higher plants, the so-called "auxins," have been the subject of much investigation; these compounds may initiate cell division and growth in the cambium and elsewhere in the plant body, and they play an essential role in normal correlation. Like many animal hormones, they are compounds of known and comparatively simple constitution (for a review cf. the recent book of Went and Thimann, 1937).

Under certain conditions the initiation of cell division and proliferation in animals and plants may take on a pathological character. Growths may originate, under local chemical or other influence, which have no relation to the normal correlation or balance of parts and which are injurious or even destructive to the whole organism. But since auxins may "cause" galls and since compounds which are normal to animals, or near derivatives of these compounds, may produce tumors,[2] the pathological problem is not to be separated from the normal physiological problem. The parallels between tumor formation and the phenomena of artificial parthenogenesis have been discussed by many pathologists (see, especially, Leo Loeb, e.g., 1911, 1915, 1920).

From the foregoing brief discussion the broad biological interest attaching to the phenomena of activation in egg cells will be apparent. Fundamental problems of the physiology of growth and development are involved. In artificial parthenogenesis the physical and chemical conditions of activation can be studied in isolated cells of uniform constitution under exactly controlled conditions. The variety of parthenogenetic methods has proved to be surprisingly great, and no detailed description will be attempted in this article. A classified list was made by Harvey in 1910.[3] Since then there has been much additional research, but the recent interest has been not so much in the discovery of new methods or the application of old methods to new cases as in the physicochemical analysis of the conditions which underlie this sudden and remarkable transformation in the properties of the egg cell.

[2] Cf. the large recent body of work on carcinogenic compounds. A recent review by S. E. Owen in *Quarterly Review of Biology*, 12 (1937), 340, discusses the biological relationships of these compounds.

[3] An extended summary of the earlier work is given in the article of E. Godlewski, Jr., "Physiologie der Zeugung," in Winterstein's Handbuch d. vergl. Physiologie, 3: Part II (1913), 805–67.

It is evident that the physiology of sperm fertilization is largely identical with that of artificial parthenogenesis, since activation is a feature of both; but in fertilization there is the additional factor of the fusion of two gametes with the accompanying genetic effects; also, the resulting activation is usually more uniform and complete than in artificial activation. Nevertheless, the latter may in favorable cases be followed by complete and apparently normal development, even in higher animals, such as the frog (Loeb and Bancroft, 1913; J. Loeb, 1920) and mammals (Pincus, 1939; Pincus and Shapiro, 1940). The initial deficiency in chromosomes may be partly responsible for the usually less favorable development in parthenogenesis, although in later stages this deficiency is apparently restored in many cases. (For chromosomes in artificial parthenogenesis of the frog see Parmenter, 1925, 1940; for a cytological study of the sea-urchin egg in early parthenogenetic development see E. B. Wilson, 1901).

The physiological analysis of the processes of artificial parthenogenesis began with the work of Jacques Loeb in 1899, although artificial activation of insect eggs had been studied earlier by Tichomiroff in 1886, and the initiation of cell division in unfertilized sea-urchin and annelid eggs by adding neutral salts to sea water (Loeb's first method) had already been accomplished by Mead and Morgan; Richard Hertwig also had obtained cell divisions in sea-urchin eggs by treatment with weak solutions of strychnine in sea water. A summary of the earlier work up to 1913 (with references to the work just cited) is given in Loeb's volume (1913). In his general discussion of factors (1913), based chiefly on his experience with the sea-urchin egg, Loeb assigned special importance to the additive or mutually supplementary effects of two distinct treatments separated in time. These were: (1) a surface effect produced by brief treatment with various, chiefly cytolytic, agents and associated with the separation of the "fertilization membrane," and (2) an internal metabolic effect involving oxygen consumption, lasting for a longer time ($\frac{1}{2}$–1 hour) and best produced by moderately hypertonic sea water (sea water with a 50–75 per cent increase in osmolar concentration produced by adding neutral salts or sugar). More recent studies have shown that the two separate treatments are not an essential feature of the activating procedure; even in sea-urchin eggs a single properly timed exposure to hypertonic sea water may give as complete an activation as a double treatment (Just, 1922*a*); and various types of single treatment (heat, acid, mechanical) have proved highly effective with other eggs (starfish, annelid, frog, mammal). Although we may concede that the internal factors immediately concerned in the initiation of development are multiple (as in any natural event), nevertheless it is an experimental fact that one simple external change of physical or chemical condition may, in many cases, so alter the conditions within the egg that it is transformed from the "resting" state, in which it sooner or later dies a "natural death" without further division or development, into an "active" state, in which it spontaneously begins and carries through more or less completely its normal cycle of development. Since the metabolic and other activity of the egg cell is thus profoundly modified by this temporary treatment, we conclude that all the factors required for development are already present or latent in the egg system but that their effective interaction is in some way prevented. Some slight change of condition is all that is needed to set in motion this process of interaction, which, once started, proceeds automatically. The critical initiatory event may be a shift of pH in the egg cortex, or on the physical side it may be an electrical or mechanical change; in some way it acts as a "releasing" factor and removes the block or hindrance to cell division and further development.

For the purposes of our present, mainly physicochemical, discussion we may describe the developmental process in general terms as consisting in a metabolic cycle of a specific kind, associated with the formation of definite structure and organization. The essential feature of the cycle is its coherence or integrated character; each step in the sequence follows automatically upon the preceding, and simultaneous processes are closely correlated or interlocked. The initiatory event in the sequence is that caused directly by the activating treatment. This event appears to be some chemical or metabolic reaction, possibly, as Loeb himself suggested, a splitting or hydrolytic reaction yielding some product or products which are utilized in later synthesis. Loeb's "corrective" process, induced by hypertonic sea water, was, in fact, regarded by him as consisting in the synthetic production of some substance necessary to further development (Loeb, 1913*a*, p. 119).

ACTIVATION IN THE STARFISH EGG

The conditions in the starfish egg indicate, more clearly than in most eggs, that activation is a progressive process, the primary stage of which consists in a metabolic reaction readily initiated by acid (or heat), which must proceed to a certain final stage if activation is to be complete. If the reaction stops short of this stage, activation is only partial; i.e., the eggs form membranes and cleave (usually slowly and irregularly) but soon cease development and disintegrate (R. S. Lillie, 1915). The most important fact about activation in this egg is its ready production by acids of all kinds. Apparently all nontoxic readily penetrating acids will activate this egg; and the rate of activation, within a considerable range of concentrations, is closely proportional to the concentration of acid (R. S. Lillie, 1914). With a given concentration of acid—e.g., 0.004 M butyric or other fatty acid dissolved in sea water—the proportion of eggs developing to a larval stage increases with duration of exposure up to a maximum and then decreases. A definite optimal duration of exposure is shown for each concentration of acid at a given temperature, and the curve relating duration of exposure to percentage of blastulae (or gastrulae) formed has a characteristic regular form, which is the same for heat activation as for acid activation. For this and other reasons it seems likely that heat activation[4] (by exposure to sea water at 30°–38° C.; R. S. Lillie, 1908, 1915) is, in reality, a special case of acid activation in which the acid is produced metabolically within the egg. Weak penetrating bases (ammonia) have no activating effect on the starfish egg. Since a partial activation resulting from brief exposure to acid or heat may be completed by a second properly timed exposure to either agent, as well as by hypertonic sea water (R. S. Lillie, 1915), the general inference seems justified that the accumulation of some reaction product to a certain level of concentration or quantity is the physiological requirement for complete activation. If the quantity of this product is too small (as with too brief exposure), the result is incomplete activation; if, on the contrary, it is produced in excess, activation is also defective, although for a different reason.

According to this interpretation, the primary or critical event in activation consists in a definite chemical reaction by which a substance necessary to development is produced within the egg. This hypothetical intermediary (comparable with a growth hormone or auxin) is called the "activating substance" (*A*). It is regarded as formed by interaction between two other substances, both of which are present in low or subthreshold con-

[4] The first successful experiments on heat activation in starfish eggs were those of Delage (1901).

centration in the resting egg. One of these (S) is a synthetic product which may be increased in quantity by treating the egg in certain ways—e.g., by hypertonic sea water in the presence of oxygen; the other (B) is freed from pre-existing combination by a simple splitting process which is initiated or catalyzed by acid (i.e., by H-ions above a certain concentration); this reaction is independent of oxygen. The final activation reaction is symbolized by the equation $S + B = A$. The production of A may accordingly be promoted by increase in the available quantity (concentration or activity) of either S or B (or of both). This simple scheme is in correspondence with the experimental fact that the egg may be completely activated in two quite different ways, either (1) by a relatively prolonged exposure to oxygen-containing hypertonic sea water—which increases S—or (2) by a brief exposure to heat or acid under either aerobic or anaerobic conditions—which increases B. The egg may also be activated by a summation of the two treatments. In the sea-urchin egg complete activation by acid alone does not occur, and a supplementary action of hypertonic sea water[5] is required. In terms of the present hypothesis, this would mean that the resting sea-urchin egg, in comparison with the starfish egg, is deficient in the S-compound. In general, the dual nature of the process in the echinoderm egg is interpreted as indicating that two different types of metabolic reaction, respectively hydrolytic and synthetic, are concerned in activation; and it is assumed that the products of these reactions interact to produce a third substance, the activating substance (A), whose accumulation leads to activation (for a fuller discussion cf. R. S. Lillie, 1934).

Influence of hypertonic sea water on activation: General.—It is a fact of much general interest that a temporary moderate increase in the osmolar concentration of the surrounding medium should have an activating influence on the unfertilized eggs of so many animals. Treatment with hypertonic solutions, physiologically indifferent apart from their hypertonicity, appears to be the most generally effective parthenogenetic method. This treatment may be supplementary, as in Loeb's "improved method" (1913*a*, chap. viii) for the sea-urchin egg, or it may, by itself, be sufficient for complete activation. In the starfish egg, as in the sea-urchin egg, a partial activation by brief treatment with some membrane-forming agent, such as fatty acid or heat, may be completed by exposure to hypertonic sea water. A similar supplementary effect, however, may be produced by a second treatment with fatty acid or heat; and it is a special peculiarity of this egg that simply prolonging the exposure to either agent, in a single continuous exposure properly timed, will cause complete activation. This is not the case in the sea-urchin egg, where simple exposure to fatty acid produces only membrane separation, followed, perhaps, by a few cleavages; the same is true of other membrane-forming agents. On the other hand, a simple exposure to oxygen-containing hypertonic sea water of appropriate concentration may activate the sea-urchin egg uniformly and completely, as has been shown by Just (1922*a*) for *Arbacia;* this is true also for *Asterias*, although in my own experience with this egg a single treatment with hypertonic sea water has not usually proved so effective as one with heat or fatty acid (cf. R. S. Lillie, 1931, p. 302).

It is evident that in the sea-urchin egg the immediate physiological effect of hypertonic sea water is different from that of fatty acid or heat. Yet in the starfish egg any one of these three agents, acting alone, may cause complete activation; accordingly, they may be called the "efficient activating agents." There are also various forms of treat-

[5] Loeb found that prolonged exposure to cold or cyanide could act, to some degree, as a substitute for hypertonic sea water.

ment whose activating effect seems never to be more than partial; shaking (Mathews, 1901), ultra-violet radiation (Lillie and Baskervill, 1922), photodynamic action (Lillie and Hinrichs, 1923), isotonic neutral salt solutions (R. S. Lillie, 1910, 1911; Dalcq, Pasteels, and Brachet, 1936), the electric current (Lillie and Cattell, 1925), and fat-solvent compounds (J. Loeb, 1913*a*; cf. p. 250) may all form membranes and induce cleavage in starfish eggs, and an occasional egg may develop to the blastula stage. But in the action of these agents there is always a deficiency that requires to be supplemented or "corrected" by an efficient agent. The purely physical action of hypertonic sea water is to abstract water osmotically from the egg, thus increasing the concentration of the egg constituents. In the absence of oxygen such a temporary loss of water seems to be physiologically indifferent; but when oxygen is present, certain secondary metabolic reactions follow which change the properties of the egg in the special manner characteristic of activation. In the sea-urchin egg some product or products of these metabolic reactions appear to be necessary for complete activation. Thus, in one of his experiments Loeb (1913*b*) found that a brief exposure of unfertilized *Strongylocentrotus* eggs to hypertonic sea water did not visibly alter the eggs or shorten their duration of life; yet for many hours after this treatment they remained in a state where they could be activated completely by a brief treatment with fatty acid. A natural inference is that some substance in which the egg was previously deficient has been formed; and this, in Loeb's view, is the basis of the "corrective" effect which he ascribed to the hypertonic treatment.

In general, according to the mass-action rule, changes of concentration resulting from addition or abstraction of water may be expected to alter the conditions of chemical equilibrium within the egg; it is, therefore, not surprising that they should have metabolic and physiological effects. Since most protoplasmic syntheses, as of proteins from amino acids, are dehydrolytic, a partial dehydration would theoretically have the effect of shifting equilibrium in the direction of further synthesis. Such an increase in certain synthetic products necessary for development might conceivably be the basis for the widespread activating influence of hypertonic solutions.

Hypertonic sea water and activation in the starfish egg.—In the starfish egg a sufficiently prolonged exposure ($\frac{1}{2}$–2 hours at 20°) to oxygen-containing hypertonic sea water alone (beginning during the prematurational period) may cause complete activation in a large majority of eggs. The duration of the optimal exposures varies with concentration, decreasing with increase of concentration within a certain range. The starfish egg also responds, like the sea-urchin egg, to after-treatment with hypertonic sea water, following a brief initial treatment with fatty acid; but this effect varies with the length of the acid treatment and is favorable only with the briefer exposures to acid (R. S. Lillie, 1931).

Eggs exposed for moderate periods (10–30 minutes at 20°) to hypertonic sea water and then returned to normal sea water without further treatment show partial activation; they form membranes and, after a period of latency (longer than in the case of complete activation), may cleave a few times, but typically they break down early without further development. If, however, within 15 or 20 minutes after return to sea water they are given a second treatment, consisting of exposure for a brief period to a weak solution of a nontoxic penetrating acid in a balanced salt solution (e.g., 0.004 M fatty acid in sea water), they exhibit the typical progressive activation with a maximal production of blastulae at a certain well-defined duration of exposure; this optimal exposure, however, is briefer than that shown by eggs with no previous hypertonic treatment. The eggs may

be described as having been "sensitized" by the hypertonic sea water to the activating influence of the acid; i.e., the activation reaction induced by the acid proceeds in the usual manner but at an increased rate. The degree of this acceleration varies (at constant temperature) with the concentration of the hypertonic sea water and the duration of the exposure. With hypertonic sea water of constant composition (100 vols. sea water *plus* 20 vols. 2.5 M NaCl) the curve relating degree of sensitization to duration of previous exposure shows a good correspondence with that of a typical bimolecular reaction (R. S. Lillie, 1934). This is consistent with the hypothesis that the sensitization (equivalent to partial activation) results from the formation of an activating substance A by the union of two precursors B and S, of which S is increased in quantity by the hypertonic treatment and B by the acid treatment.

TABLE 1

June 29, 1934. Eggs from several starfish were exposed during the prematurational period to hypertonic sea water for 20 minutes at 20°–21° C. and returned to normal sea water. After 18 minutes in sea water, they were transferred to a 0.004 M solution of butyric acid in sea water; from this solution portions were returned at 1-minute intervals to normal sea water. Next day the proportion of eggs developing to a blastula stage with each duration of exposure was determined.

Concentration of Hypertonic Sea Water (Vols. 2.5 M Na-Ca Added to 100 Vols. Sea Water)	Duration of Optimal Exposure and Approximate Percentages of Eggs Developing to Blastula Stage
A. 0 (control)	6–7 min. (85–90 per cent)
B. 10	5–6 (70–80)
C. 20	*ca*. 4 (50–60)
D. 30	*ca*. 4 (25–35)

Influence of concentration.—In experiments carried out chiefly during the summers of 1934 and 1935, I have studied in some detail the relation between the concentration of the hypertonic sea water and the degree of sensitizing action. The sea water was made hypertonic by the addition of a strong partly balanced salt solution consisting of 95 volumes of 2.5 M NaCl *plus* 5 volumes of 2.5 M $CaCl_2$ (called, in the following, "2.5 M Na-Ca").

There is a lower limit of effective hypertonicity corresponding approximately to the mixture of 10 volumes of 2.5 M Na-Ca *plus* 100 volumes of sea water. The mixture of 5 volumes of 2.5 M Na-Ca *plus* 100 volumes of sea water has no apparent effect. In experiments with additions ranging from 10 to 30 volumes of 2.5 M Na-Ca to 100 volumes of sea water the effect is distinct, and its rate (as shown by the sensitization produced by a constant exposure) increases with concentration. Hypertonicity beyond this range is quickly injurious to the eggs.

A typical protocol (June 29, 1934), as given in Table 1, shows the general manner in which the rate of activation increases with increase in the concentration of hypertonic sea water. In this experiment the control eggs (A), treated with 0.004 M butyric acid alone, showed optimal development with 6–7 minutes' exposure. The effect of the previous stay in hypertonic sea water is seen in a shortening of the optimal exposure; this effect increases with concentration.

Table 2 summarizes the results of a considerable number (33 in all) of similar experi-

ments carried out during these two summers. The arithmetical means of the optimal activation times of experiments conducted under similar conditions are given. Usually the optimal exposure to 0.004 M butyric acid (in sea water) at 20°–21°, without any previous treatment with hypertonic sea water, lies between 6 and 8 minutes, varying somewhat with the animals and with the season of the year. Often in a single experiment the time curve of activation may have a flattened top; i.e., two (or even three) exposures separated by intervals of 1 minute (e.g., 6 and 7 minutes) may appear equally favorable, as in Experiment *A* above. In computing the averages, the optimal exposure in any such case is taken as the mean of the two (or more) equally favorable exposures (i.e., 6.5 minutes in the example cited). In all experiments the exposures to hypertonic sea water lasted 20–21 minutes, and the temperature was 20°–22°.

TABLE 2

In all experiments the eggs were exposed during the prematurational period to the hypertonic sea water at room temperature (20°–22° C.) for 20–21 minutes; then, after 15–20 minutes in sea water, they were transferred to 0.004 M butyric acid in sea water at the same temperature. Portions were then returned to normal sea water at intervals of 1 minute. (The number of separate experiments with each concentration of hypertonic sea water is indicated in brackets in the first column. Probable errors by Peter's approximation formula.)

Hypertonic Sea Water (Vols. 2.5 M Na-Ca Added to 100 Vols. Sea Water)	Average Optimal Exposure for Control Eggs Treated with Acid Alone (Minutes)	Average Optimal Exposure for Eggs Treated also with Hypertonic Sea Water	Percentage of Decrease in Optima with Hypertonic Treatment (Nearest Whole Number)
5 (5)	6.0±0.51	5.9±0.47	0
10 (7)	6.5±0.40	5.7±0.37	12
15 (9)	7.5±0.46	6.1±0.39	18
20 (6)	8.2±0.44	5.9±0.34	28
25 (3)	9.2±0.14	6.2±1.0	33
30 (2)	6.5±0	4.2±0.21	35

As already mentioned, Loeb's view of the dual nature of the activation process was based on the observed difference in the mode of action of the two treatments, both of which he found necessary for complete activation in the sea-urchin egg. One of these acts superficially, is rapid, and is independent of respiration; the other (called "corrective") occurs while the egg is in the hypertonic sea water; it is more prolonged and requires the presence of oxygen. The fact that hypertonic sea water, acting alone, may activate sea-urchin eggs completely has been considered inconsistent with Loeb's interpretation (Just, 1922*a*), but such a criticism seems hardly justified. In the starfish egg, also, the mutually supplementary action of the two treatments is shown; yet either, acting alone, may cause complete activation. There is, however, no inconsistency if activation is regarded as dependent in the union of two substances, *S* and *B*, both of which are present in low or subthreshold concentration at the site of activation and in equilibrium with the activating substance, *A*, formed by their combination. According to

this view, the accumulation of A to the critical level required for complete activation is the essential feature of the process. Experiment shows that activation is a progressive process which may be stopped at any stage short of completion and later be renewed. The incompletely activated egg behaves as if it were deficient in some material necessary for development. By the mass-action rule a sufficient increase in either S (formed by hypertonic sea water) or B (freed by some splitting process promoted by acid) would lead to a sufficient production of A, and so to activation. The sequence of metabolic reactions started by activation is necessarily complex, since their consequence is development; but it seems clear that the initial or releasing reaction of this sequence may require only a slight change of equilibrium. This primary or critical change is to be regarded as definitely localized in the cytoplasm of the egg, in a region which apparently is to be identified with the most superficial layer or cortical region (Chambers, 1921*a*, 1921*b*). Mrs. Harvey's recent experiments on parthenogenetic merogony (E. B. Harvey, 1936, 1940) furnish additional and crucial evidence that activation is a cytoplasmic reaction independent of immediate nuclear influence.

Rate of activation reaction as directly influenced by hypertonic sea water.—The purely physical effect produced by hypertonic sea water is withdrawal of water. It should be noted that this effect must occur most rapidly in the superficial region of the egg, corresponding to the so-called "cortical region." Although 10–15 minutes elapse before the whole egg reaches osmotic equilibrium with its surroundings (cf. Stewart and Jacobs, 1932), this entire period corresponds to the time required for an osmolar concentration equal to that of the hypertonic sea water to be attained within the whole volume of the egg. During the time occupied by the osmotic movement of water, there is a gradient of concentration from the external to the internal regions of the egg, with the highest concentration at the surface. Accordingly, when the egg is placed in the hypertonic sea water, a thin layer of protoplasm immediately inside the plasma membrane will almost instantly attain osmotic equilibrium with the surroundings, and a flow of water from within outward will continue as long as the internal regions are more dilute than the external. Transfer of water then ceases, and the whole system is in equilibrium.

The activating effect of such a temporary increase of concentration is dependent on oxidative metabolism, as we have seen, and is relatively gradual. Eggs remaining in this partly dehydrated condition for 10–20 minutes show an increased rate of activation when, after a brief interval in sea water (during which they regain their normal water content), they are placed in acid-containing sea water (sensitization effect; Table 1). If, however, they are placed in this solution directly, i.e., without the interval in sea water—or while the egg cortex is still in a water-depleted state—the acceleration is much less, or activation may even be retarded instead of accelerated. Apparently, the water content of the egg at the actual time of activation influences the rate of the reaction directly. Conversely, it is found that the rate of activation is increased by addition of water to the egg, as when activation occurs in dilute sea water or after brief exposure to dilute sea water (see below). It may seem surprising that, while an exposure of 20 or 30 minutes to hypertonic sea water brings the eggs into a state in which, after regaining their normal water content, they show an accelerated response to acid or heat, the activation reaction itself is retarded if it occurs *while* the eggs are still in the partly dehydrated condition. Apparently this means that a decrease in the water content of the protoplasmic system has in itself a delaying effect on the progress of the activation reaction. Since dilution has the opposite effect, the inference seems natural that the rate

at which the precursors (symbolized by *B* and *S* of our scheme) unite to form the activating compound (*A*) is determined (in part) by the water content, i.e., the structural density, of the protoplasm at the site of the reaction, the cortical region. Apparently, increased density slows diffusion and hence rate of interaction, while decreased density has the reverse effect.

To illustrate this directly retarding effect of partial dehydration, an experiment described in a former paper may be cited (R. S. Lillie, 1934, p. 372). Two portions of the same lot of eggs transferred simultaneously from normal sea water to two 0.004 M solutions of butyric acid—one (*A*) in normal sea water, the other (*B*) in hypertonic sea water (100 vols. sea water *plus* 20 vols. 2.5 M Na-Ca)—showed optimal exposures (with 80–90 per cent of blastulae) at 8 minutes in *A* and at 14 minutes (or longer) in *B*. This result is typical and has been confirmed many times under a variety of conditions.

TABLE 3

July 17, 1935. Mixed eggs from several starfish were used; 95 per cent or more maturated; sperm-fertilized controls are normal. All treatments were during prematurational period.

Treatment	Result
A. (Control.) Eggs exposed to 0.004 M butyric acid in sea water	Optimal exposure at 5–6 min. (*ca.* 50 per cent blastulae)
B. Eggs exposed to hypertonic sea water (100 vols. sea water *plus* 10 vols. 2.5 M Na-Ca) for 25 min.; then to normal sea water for 17 min.; then to 0.004 M butyric acid in sea water	Good activation with optimum at 4–5 min. (70–80 per cent blastulae)
C. Same treatment with hypertonic sea water, but eggs were transferred directly to 0.004 M butyric acid in sea water	Also good activation with optimum at 6 min. (70–80 per cent blastulae)

In experiments of the type summarized in Table 2, the interval in normal sea water allows the eggs time to regain their normal water content; activation is then found to be definitely more rapid than in untreated eggs. The experiment described in Table 3, in which a less concentrated hypertonic sea water (100 vols. sea water *plus* 10 vols. 2.5 M Na-Ca) was used, shows the effect of omitting the interval in normal sea water. In this experiment the eggs treated first with hypertonic sea water and then returned to normal sea water for an interval (*B*) show the usual increased rate of activation; while those transferred directly to the acid-containing sea water (*C*) show no acceleration but rather a slight retardation. Experiments with more concentrated hypertonic sea water (100 vols. sea water *plus* 20 vols. 2.5 M Na-Ca) gave the same result; but in such cases the effect of direct transfer to the acid-containing sea water is injurious, probably because of the greater osmotic change, and few eggs develop to the blastula stage.

In experiments of this type (*C*) the activation reaction begins while the eggs are still in a partly dehydrated state, and the retarding effect of this condition shows itself in a

partial counteraction of the acceleration which otherwise would be expected from the action of the hypertonic sea water. But since the osmotic entrance of water into the cortical layer begins immediately after the eggs are transferred to the acid-containing sea water, the degree of counteraction is relatively slight. When this osmotic change is avoided, as by bringing the eggs from hypertonic sea water directly into a solution of acid *made up in the same medium*, the retardation is much greater, and it increases rapidly with increase in hypertonicity. Many experiments of this kind have been carried out during several seasons; and the results, while showing considerable quantitative variation, have been entirely uniform as regards the direction of the effect.

The experiment described in Table 4 is typical and shows the increase of retardation with increase in hypertonicity. In this experiment the eggs were exposed to neutral hypertonic sea water for ½ hour before being exposed to acid. In other experiments, how-

TABLE 4

August 5, 1936. Good lot of eggs; great majority show normal maturation and develop normally on fertilization.

Treatment	Result
A. (Control.) 0.004 M butyric acid in sea water. Temperature 22° C.	Optimum at 5 min. *Ca.* 90 per cent of mature eggs form blastulae
B. Eggs exposed to hypertonic sea water (100 vols. sea water *plus* 10 vols. 2.5 M Na-Ca) for 32 min., then to 0.004 M butyric acid in the same solution	Retardation distinct but moderate; optimum at 6 min., with *ca.* 50 per cent blastulae
C. Treatment as in *B*, but the hypertonic sea water is more concentrated (100 vols. sea water *plus* 20 vols. 2.5 M Na-Ca)	Marked retardation; optimum at 9 min. with 80–90 per cent blastulae; 70–80 per cent at 11 min. (the longest exposure)

ever, the duration of the previous hypertonic treatment was found, somewhat unexpectedly, to make little or no difference in the rate of activation in the acid-containing solution. An experiment comparing the influence of brief and longer exposures to hypertonic sea water is given in Table 5. As already described, retardation is shown if eggs are transferred directly from normal sea water to acid-containing hypertonic sea water, although typically the retardation observed under such conditions is less than with a previous hypertonic treatment. The relative absence of a time influence is understood if the activation reaction is considered as having its site in the cortical layer of the egg, where the osmotic loss of water occurs most rapidly.

The average retardations (expressed in terms of increase in optimal exposure) observed in a considerable number of experiments during 1936 and 1937, with previous exposures of 16–35 minutes to hypertonic sea water of two concentrations, are shown in Table 6. The concentration of butyric acid was 0.004 M in all cases; and the temperature of the acid solutions, 20°–22°. The number of experiments with each concentration is indicated in the first column, in brackets.

From these experiments it is clear that the activation reaction induced by fatty acid

proceeds at a decreased velocity in eggs which are already in a state of partial dehydration under the influence of hypertonic sea water. In the terms of our hypothesis the velocity of the reaction $S + B = A$ is decreased. Experiments on the heat activation of starfish eggs also show a decreased rate of reaction when the eggs are exposed to activating temperatures while immersed in hypertonic sea water. Thirteen experiments of this kind were performed during the summers of 1938 and 1939; the eggs were exposed (A) to normal and (B) to hypertonic sea water at 32° simultaneously and under otherwise similar conditions, without previous hypertonic treatment. The sudden transfer from normal sea water at 20° to hypertonic sea water at 32° appears to be injurious to the eggs, and blastulae were usually fewer than in the control (normal sea water at 32°). Lower concentrations of hypertonic sea water were used, with additions of 2.5 M Na-Ca ranging between 1.5 and 10 volumes to 100 volumes of sea water. Under these conditions a large

TABLE 5

July 23, 1937. Most eggs show normal maturation; sperm-fertilized controls are normal.

Treatment	Result
A. (Control.) 0.004 M butyric acid in sea water. Temperature 21°–22° C.	Optimum at 8 min.; *ca.* 50 per cent of mature eggs form blastulae
B. Brief treatment of *ca.* 1 min. with hypertonic sea water (100 vols. sea water *plus* 20 vols. 2.5 M Na-Ca); thence to 0.004 M butyric acid in the same medium	Retarded activation with optimum at 11 min. (*ca.* 50 per cent blastulae)
C. 20 minutes in the same hypertonic sea water as in *B;* otherwise similar	Retarded activation with optimum at 10–11 min. (20–30 per cent blastulae)

proportion of eggs may form blastulae; and retardation, while moderate, is distinct, especially in the mixtures with additions of 5–10 volumes of 2.5 M Na-Ca. Membrane separation is especially subject to hindrance under these conditions; typically, about 3 minutes' exposure to normal sea water at 32° is required to form membranes in a ma-

TABLE 6*

Hypertonic Sea Water	Average Optimum for Control (Minutes)	Average Optimum with Hypertonic Treatment	Percentage of Increase in Duration of Optimum over Control
100 vols. sea water *plus* 10 vols. 2.5 M Na-Ca (4)....	5.6±0.55	6.9±0.55	23
100 vols. sea water *plus* 20 vols. 2.5 M Na-Ca (17)....	7.5±0.27	11.3±0.37	50

* In 1 experiment with less concentrated hypertonic sea water (100 vols. sea water *plus* 5 vols. 2.5 M Na-Ca) no retardation was shown. In 2 experiments with an intermediate concentration (100+15) the optima for the controls were 9 and 5 minutes, respectively, for the treated eggs 13 and 7 minutes.

jority of eggs (R. S. Lillie, 1915; cf. p. 274); but in the hypertonic mixtures with 5 volumes of 2.5 M Na-Ca the time was prolonged to 5 minutes or more, and even the mixture with 1.25 volumes had an appreciable effect.

Influence of dilute sea water on activation.—In contrast to hypertonic sea water, a temporary exposure of starfish eggs to moderately dilute (hypotonic) sea water has no definite effect in altering the rate of subsequent activation by fatty acid. There is no evidence of retardation, i.e., of an effect opposite in character to the typical acceleration or sensitization following temporary hypertonic treatment. No immediate activating effect is seen, not even membrane formation. Apparently the intake of water is perfectly reversible; and the eggs remain unaffected in their ability to respond later to artificial activation or sperm fertilization. This absence of effect was noted in my 1934 paper and has been repeatedly confirmed in more recent experiments.

It should be noted, however, that in the eggs of some other species of animals hypotonic solutions have proved to be remarkably effective parthenogenetic agents. This is notably the case in the egg of the annelid *Urechis*, as recently shown by Tyler (1931*a*, 1931*b*, 1932); and recently Pincus and Shapiro (1940) have found dilute serum or hypotonic balanced salt solution highly favorable with mammalian eggs.

In the experiments with temporary hypotonic treatment dilutions of sea water ranging between 90 volumes per cent (90 vols. sea water *plus* 10 vols. distilled water) and 60 volumes per cent were used, with exposures of 20–35 minutes, followed after an interval of 10 or 15 minutes in sea water by exposure to 0.004 M butyric acid in sea water. Since the results were uniformly negative, they may be summarized briefly. With the higher dilutions (60–70 vols. per cent), a slight acceleration (shortening of the optimal exposures to acid) was observed in several experiments, but the lower dilutions appeared quite indifferent. Some slight activating or sensitizing effect seems indicated in the higher dilutions, although no fertilization membranes were formed; but, in general, the absence of any decided influence in either direction is in striking contrast with the definite action of the hypertonic medium.

On the other hand, if starfish eggs are activated by fatty acid or heat *during* the immersion in dilute sea water, the rate of activation is definitely accelerated; this effect is appreciable in low dilutions and increases rapidly as the dilution is increased. In typical experiments of this kind the eggs are first exposed to dilute sea water and then transferred directly to a solution of butyric acid made up in the same medium. If, after the preliminary exposure to dilute sea water, the eggs are transferred to a solution of the butyric acid in normal sea water, they also show an increased rate of activation, but the effect is relatively slight (as in the analogous experiments with concentrated sea water) and is distinct only with the higher dilutions. This is shown in the experiment described in Table 7. Activation is definitely but moderately accelerated after the 70 volumes per cent dilution, and still more so after the 60 volumes per cent, but the higher dilution is injurious.

If, however, the eggs, after the exposure to dilute sea water, are transferred directly to 0.004 M butyric acid in the same dilute sea water, acceleration is much greater. The experiment described in Table 8 will illustrate. In this experiment activation occurs at twice the rate in the dilute as in the normal sea water, with the same lot of eggs and the same previous exposure to dilute sea water.

As in the case of hypertonic sea water, the duration of the previous stay in the dilute

sea water has little influence on the rate of activation in the acid-containing dilute sea water. This was shown in experiments in which the eggs were previously exposed to dilute sea water for periods varying from 1 minute (about the least time possible with the

TABLE 7

July 13, 1931. Most eggs (75–85 per cent) show normal maturation and develop normally on fertilization.

Treatment	Result
A. (Control.) Eggs exposed to 0.004 M butyric acid in sea water. Temperature 22° C.	Good activation with optimum at 6 min. (65–75 per cent blastulae)
B. Eggs in 80 vols. per cent sea water for 28 min.; thence to 0.004 M acid in sea water as in *A*	Very like control; optimum at 6 min. (70–80 per cent blastulae)
C. Eggs in 70 vols. per cent sea water 28 min.; thence to acid sea water as above	Moderate acceleration; optimum at *ca*. 5 min. (55–65 per cent blastulae; at 6 min. *ca*. 50 per cent)
D. Eggs in 60 vols. per cent sea water 28 min.; thence to acid sea water as above	Treatment is injurious; relatively few eggs form blastulae; optimum at 4 min. (*ca*. 20 per cent blastulae)

TABLE 8

July 30, 1936. Eggs show 90 per cent maturation and develop normally after fertilization.

Treatment	Result
A. (Control.) Eggs exposed to 0.004 M butyric acid in sea water (22.5° C.)	Typical activation with optimum at 6–7 min. (65–75 per cent blastulae)
B. Eggs in 75 vols. per cent sea water for 32 min.; then to 0.004 M butyric acid in normal sea water	Typical activation with moderate acceleration; optimum at 4–5 min. (80–90 per cent blastulae)
C. Similar to *B*, but with 0.004 M butyric acid in 75 vols. per cent sea water	Marked acceleration with optimum at 2–3 min. (60–70 per cent blastulae; eggs are overexposed at 4 min. and form no blastulae)

necessary manipulation) to 35 minutes. A typical protocol is given in Table 9. The rate of activation is greatly increased with both the brief and the longer exposures but is only slightly greater in the latter case. Eggs transferred directly from normal sea water to the 0.004 M solution of butyric acid in 80 volumes per cent sea water also show an increased rate of activation, but this treatment is injurious to the eggs, and relatively few reach the blastula stage.

During the summers of 1936 and 1937 a considerable number of experiments (29 in all) were made in which eggs were exposed for longer periods, of 15–36 minutes, to dilute sea water and then activated by direct transfer to 0.004 M butyric acid in the same medium. Omitting four of these (in which the proportion of good eggs was small), the average optimal durations of exposure in four dilutions were as given in Table 10, third column.

TABLE 9

July 28, 1937. Mixed eggs from 2 starfish; 80–90 per cent maturation. Normal development on fertilization.

Treatment	Result
A. (Control.) Eggs exposed to 0.004 M butyric acid in normal sea water (21°–22° C.)	Typical activation with optimum at 6–7 min. (70–80 per cent blastulae)
B. Eggs in 80 vols. per cent sea water for 1 min., 5 sec.; then to 0.004 M butyric acid in 80 vols. per cent sea water	Optimum at 3 min. (*ca.* 50 per cent blastulae)
C. Eggs in 80 vols. per cent sea water for 20 min.; then to 0.004 M butyric acid in 80 vols. per cent sea water	Optimum at 2–3 min. (2 min.: 60–70 per cent; 3 min.: 70–80 per cent blastulae)

TABLE 10

The first column gives the dilutions used, with the corresponding number of experiments in brackets;* the second column, the average optima for the controls (0.004 M butyric acid in normal sea water); the third column, the average optima in dilute sea water; the fourth column, the average decrease in these optima, expressed as percentages of the optima in the controls.

Dilution (Vols. Per Cent Sea Water)	Optima in Controls (Minutes)	Optima in Dilute Sea Water	Percentage of Decrease
90 (5).....	6.4±0.66	4.6±0.39	28
85 (5).....	6.5±0.67	4.1±0.50	37
80 (7).....	6.6±0.27	3.3±0.26	50
75 (8).....	8.0±0.44	3.5±0.30	56

* Two similar experiments with 70 vols. per cent sea water gave optima in the controls of 8 minutes and 6 minutes, and in the dilute sea water of 3 minutes and 2 minutes, an average decrease of 64 per cent.

The preliminary exposure to dilute sea water, even if brief, is an important part of the treatment, since a direct transfer from normal sea water to the acid-containing dilute sea water is highly injurious. In a typical experiment (July 23, 1936), eggs which yielded 80–90 per cent of blastulae with an optimal exposure of 10 minutes to 0.004 M butyric acid in sea water gave only 2 or 3 per cent, with an optimum at 4 minutes, when transferred directly to 75 volumes per cent sea water containing the same concentration of acid. Eggs of the same lot previously exposed for ½ hour to dilute sea water showed also

an optimum at 4 minutes, but the great majority (65–75 per cent) formed blastulae. It would seem that when the action of the acid occurs simultaneously with the sudden osmotic entrance of water into the surface layer of the egg an injurious effect results which is absent when the surface layer is already diluted before the entrance of the acid. As in the analogous case of hypertonic sea water, the rapidity with which the dilution effect appears is evidence that the activation reaction occurs in the cortical layer of the egg.

Heat activation is also accelerated when the eggs are warmed to 32° in dilute sea water, either with or without a previous exposure to the dilute sea water; but the degree of acceleration has proved to be somewhat less than with acid activation. A typical experiment with direct transfer from normal sea water to warm sea water, both normal and dilute, is given in Table 11.

In a typical experiment with eggs previously exposed to a somewhat highly dilute sea water (65 vols. per cent) for 23 minutes and then transferred to the same medium at 32°, a well-defined optimum was shown at 3 minutes (*ca.* 50 per cent blastulae); while in the

TABLE 11

June 22, 1938. Eggs from 2 starfish; more than 90 per cent maturate. Fertilized control normal.

Treatment	Result
A. (Control.) Normal sea water at 32° C.	Optimum at 5–6 min. with 80–90 per cent blastulae
B. 90 vols. per cent sea water at 32°	Optimum at 4–5 min. with 70–80 per cent blastulae
C. 85 vols. per cent sea water at 32°	Optimum at 4 min. with 70–80 per cent blastulae

control (normal sea water at 32°) the optimum was 5 minutes. In the warm normal sea water the time required to form membranes in a majority of eggs was 3 minutes; this was shortened to 2 minutes (or less) in the dilute sea water. The same facilitation of membrane separation was found uniformly in all the experiments in which eggs were warmed in the dilute medium. In general, all the typical effects which result from treatment with activating agents (acid as well as heat) appear more rapidly in the dilute medium. This is true, for example, of the injury following exposures longer than the optimal. Thus, in the experiment just cited the eggs warmed to 32° in the dilute sea water for 5 minutes showed all the effects of overexposure (early disintegration with no blastulae formed), while the same exposure was optimal for the eggs warmed in normal sea water. It is as if a single metabolic reaction, on which the other physiological effects of activation depend, proceeds at an increased rate in the diluted protoplasmic layer.

It should again be noted that it is only while the egg protoplasm is in the diluted state that this facilitation is observed. Experiments in which eggs are returned from the dilute to normal sea water before treating with acid show the complete reversibility of the effects of dilution. It appears, therefore, that the velocity of the activation reaction is determined (in part) by the water content, i.e., the structural density, of the egg protoplasm and varies reversibly with this. The opposite effect, retardation, is seen in the experiments where the eggs are activated in hypertonic sea water. Hypertonic sea water,

however, has an additional special activating effect in the presence of oxygen; this action, as already described, proceeds gradually and is referred above to an oxygen-dependent metabolic reaction by which a special substance, S, is synthesized. But in the absence of oxygen (or presence of cyanide) the effect of hypertonic sea water appears to be a purely physical and osmotic one and to be completely reversible in the same sense as the effect of dilute sea water. The fact that simply changing the water content of the egg alters the velocity, but not the essential character, of the progressive reaction by which the egg is brought from the resting into the active state is evidence that the structural density of the protoplasmic gel in the egg cortex controls the rate of union of the reactants (the hypothetical B and S of our scheme) to form the activating substance (A). It is known that the rate of diffusion of large molecules in gels—a factor which limits their rate of chemical interaction under such conditions—varies with the density of the gel in the same general manner (cf., e.g., Kraemer, 1931, p. 1711; Freundlich, 1923, p. 727).

Other conditions influencing the velocity of the activation reaction in starfish eggs.—Temperature is a general physical condition which appears to influence the rate of activation in two different ways. At a lower range of temperatures, up to 20°, activation in butyric acid solutions in sea water shows the usual chemical type of temperature coefficient (Q_{10} = *ca.* 3); but at higher temperatures the Q_{10} values increase rapidly (R. S. Lillie, 1917), indicating apparently the superposition of an incipient heat-activation effect. This effect, in its turn, is probably to be referred to the production of additional acid within the egg. The activating effect of cold on the starfish egg (Greeley, 1902) is a special phenomenon peculiar to this egg, referable possibly to some structural (e.g., colloidal) change in the egg cortex, analogous to that caused by mechanical treatment.[6] This structural change initiates the activation reaction, presumably through the production of acid. Activation by butyric acid at low temperatures (2°–6°) is more rapid than would be expected from a consideration of temperature conditions alone (R. S. Lillie, 1917), indicating a summation effect analogous to that observed at high temperatures.

The velocity and character of the activation reaction in acid sea water are not immediately changed by lack of oxygen or presence of cyanide; but, if the eggs are kept under anaerobic conditions for some time before they are activated, an acceleration is shown, apparently as a result of increased acid production within the egg (R. S. Lillie, 1931). Activation by heat or acid may be influenced by other types of chemical condition; thus, certain compounds may increase the rate of activation by their mere presence, independently of any direct activating influence of their own. An interesting example, observed some years ago, is the acceleration in weak solutions of ethyl alcohol (R. S. Lillie, 1914, 1917). In sea water containing 2–3 volumes per cent of alcohol the velocity of both heat and acid activation is approximately doubled. Activation by cold (2°–4°) is also promoted by alcohol in a striking way. Since alcohol alone causes no appreciable activation in these concentrations, some physical effect (such as displacement of reactants from adsorption, alteration of viscosity, etc.) would appear to be involved; but the phenomenon requires further investigation.

Activation in mixtures of sea water and isotonic dextrose solution.—Acids have a special relation to activation in the starfish egg, as we have seen; and since the same sequence of physiological effects is produced by heat activation as by acid activation, the former is,

[6] Pincus and Shapiro (1940) found that rabbits' eggs could be activated very effectively by cooling (20° *in vitro*, or surrounding the Fallopian tube with a cold-water jacket).

in all likelihood, a special case of acid activation. In many cells acids are formed in large quantity as a result of moderate rise of temperature—for example, in the heat rigor of muscle; the typical source of the stronger acids formed under such conditions, as well as in normal metabolism (chiefly lactic, pyruvic, and other oxy-acids), is the breakdown of carbohydrate (glycolysis). Empirically, we find that the activation of starfish eggs in 0.004 M butyric acid in sea water at 20° proceeds at very nearly the same rate as heat activation in normal sea water at 32°; in both cases the optimal exposures lie typically between 6 and 9 minutes. The most noticeable difference is that in heat activation there is a distinct lag in membrane formation, which usually requires about 3 minutes' exposure to 32°, while in activation with 0.004 M butyric acid at room temperature the same effect is produced in 1 minute or less. This difference probably represents the time required for the metabolically produced acid to reach an effective concentration. According to the present hypothesis, the acid is regarded as producing its activating effect by setting free (e.g., by catalysis in some hydrolysis reaction) one of the reactants (*B*) of the activation reaction. The acid appears to act at a rate which is closely proportional to the increase of H-ion concentration within the egg above a certain level, since it is found that solutions of different penetrating acids which activate at the same rate (at constant temperature) have approximately the same cH values (R. S. Lillie, 1927).

Since carbohydrate readily gives rise to acid in metabolism, it might be expected, if activation is, in fact, associated with metabolic acid production (cf. p. 260), that artificial increase in the carbohydrate content of the egg would increase the rate of activation by an additive kind of effect. The presence of dextrose in the medium in high concentration is nontoxic; the eggs undergo maturation, fertilization, and development to larval stages in mixtures of equal volumes or more of isotonic dextrose solution with sea water. Experimentally, it was found that if eggs are activated by acid dissolved in such mixtures, the rate of activation is markedly increased. Previous exposure to the neutral dextrose sea-water mixture is unimportant; the acid and dextrose must be present simultaneously in the medium. This is shown in the experiment described in Table 12. The rate of activation is seen to be approximately doubled in the dextrose sea-water mixture, and the same acceleration is shown whether the eggs have previously been exposed to the dextrose solution (*C*) or not (*D*). But eggs previously exposed to dextrose sea water and then activated by acid in sea water (*B*) show the same rate of activation as the control. This accelerating influence of dextrose was shown without exception in 10 similar series of experiments in the summer of 1940. The absence of any effect from the previous exposure to dextrose indicates nonpenetration (or slow penetration) of this substance into the resting egg. Activation is apparently associated with increased permeability to dextrose.

The degree of the acceleration increases with the concentration of dextrose (up to 75 vols. per cent). Table 13 shows this relation (one of three experiments giving the same result). The eggs were transferred directly from sea water to the dextrose–sea-water mixtures containing the acid.

Experiments were also performed with pure isotonic dextrose solution; but in such solutions the eggs die rapidly, because of the absence of salts, and no activation is possible. Fertilization in this solution is also impossible.

Experiments on heat activation in dextrose–sea-water mixtures have been few so far, but in two experiments in which the control eggs exposed to sea water at 32° yielded a fair proportion of blastulae (40–50 per cent with 6 minutes' exposure), eggs warmed to

32° in 50 volumes per cent and 75 volumes per cent dextrose sea water gave almost the same result, except for interference with membrane separation. This relative absence of

TABLE 12

June 19, 1940. Eggs from several starfish were used. Maturation and fertilization were normal in the great majority.

Treatment	Result
A. (Control.) 0.004 M butyric acid in sea water. Temperature 21° C.	Optimum at 6 min. with 80–85 per cent blastulae (7 min. slightly less favorable)
B. Eggs kept for 22 min. in 75 vols. per cent dextrose–sea-water solution (75 vols. M-dextrose *plus* 25 vols. sea water); then to 0.004 M butyric acid in sea water	Similar to *A;* optimum at 6–7 min. (70–80 per cent blastulae)
C. Eggs in 75 vols. per cent dextrose–sea-water solution as in *B;* then to 0.004 M butyric acid in the same solution	Optimum at 3–4 min. (65–75 blastulae)
D. Eggs transferred directly from sea water to 0.004 M butyric acid in 75 vols. per cent dextrose–sea-water solution	Optimum at 3 minutes with 65–75 per cent blastulae; somewhat fewer blastulae at 4 min. (*ca.* 50 per cent)

TABLE 13

June 25, 1940. Eggs chiefly from one starfish. Maturation, fertilization and development are normal in most eggs.

Treatment	Result
A. (Control.) 0.004 M butyric acid in sea water. Temperature 17°–18° C.	Longest exposure (12 min.) is below optimum at this low temperture, and blastulae are few
B. 0.004 M butyric acid in 25 vols. per cent dextrose–sea-water solution	Optimum at 11 min. (longest exposure of series) with 50–60 per cent blastulae
C. 0.004 M butyric acid in 50 vols. per cent dextrose–sea-water solution	Optimum at 9–10 min. with 70–80 per cent blastulae
D. 0.004 M butyric acid in 75 vols. per cent dextrose–sea-water solution	Optimum at 8–9 min. with 70–80 per cent blastulae

effect would indicate that acceleration under these conditions, if present, is less than in the case of acid activation, but further experimentation is needed.

In the experiments just described the eggs were exposed to dextrose sea water and activated during the prematurational period. Under normal conditions in sea water the

response to artificial activation, as well as to sperm fertilization, falls off rapidly after the polar bodies are separated (R. S. Lillie, 1908); few, if any, eggs form blastulae if exposed to heat or acid after maturation is completed. If, however, the eggs are kept in dextrose–sea-water mixtures, instead of in sea water, for several hours after removal, they are found to retain, to a remarkable degree, the response to acid activation. This effect is illustrated in Table 14. Under these conditions, there would appear to be some intake of dextrose by the eggs, in contrast to the prematurational eggs. Maturation proceeds normally in such mixtures. A similar, though less favorable, result was obtained in experiments with 75 volumes per cent of dextrose sea water. Experiments of this kind with heat activation have not yet been tried.

TABLE 14

June 28, 1940. Eggs from one starfish; normal maturation (>90 per cent) and normal development after fertilization.

Treatment	Result
A. (Control.) Eggs exposed (during prematurational period) to 0.004 M butyric acid in sea water (19° C.)	Good activation with optimum at 10 min. (90 per cent blastulae)
B. Eggs in sea water for 35 min. after removal; then to 50 vols. per cent dextrose–sea-water solution for 3 hr. 11 min.; then to 0.004 M butyric acid in sea water	A large proportion (from a third to a half of the eggs) form blastulae at all exposures from 2 min. to 11 min. No well-defined optimum
C. Similar treatment with dextrose–sea-water solution, but eggs exposed to 0.004 M butyric acid in 50 vols. per cent dextrose–sea-water solution	Result similar to that in *B*, but the proportion of blastulae falls off at 8 min. None at 11 min.
D. Eggs left in sea water until time of activation (total stay in sea water *ca.* 4 hr.); then to 0.004 M butyric acid in 50 vols. per cent dextrose–sea-water solution	Blastulae numerous, but fewer than in *B* or *C*, with optimum at 6 min. (20–25 per cent). Few or none with exposures of 7–11 min.

A natural inference is that the normal loss of activability in the starfish egg after maturation results from depletion of carbohydrate. It is possible, however, that other factors may enter, such as the effect of decrease in the external salt concentration per se. According to Schechter (1937; *cf.* also Lindahl, 1936, p. 243; 1938), decrease in calcium greatly prolongs the fertilizable life of *Arbacia* eggs; and I have myself found that unfertilized *Arbacia* eggs live much longer in dextrose–sea-water mixtures than in pure sea water. The part played by concentration of salts, as such, requires further investigation.

CHEMICAL AND OTHER FACTORS IN ACTIVATION: GENERAL

The problem of the nature of the physicochemical, metabolic, and physiological factors which transform the egg from its inert or resting state into an active cell which auto-

matically divides and passes through a complex cycle of development is a baffling one.[7] As already indicated, this problem forms part of the problem of the initiation of growth and differentiation in resting cells or tissues in general. The statement that activation removes a block to further activity merely expresses the problem in another form, i.e., it recognizes the pre-existence of latent or potentially active factors; the question is what releases these from quiescence—or "activates" them. To say that the course of metabolism is altered is a truism. There are evident parallels between the activation of egg cells and the various other biological manifestations of stimulation and response; but in making this comparison, we are doing no more than emphasizing the importance of fundamental conditions common to all reactive living systems. The methods of artificial parthenogenesis are described as nonspecific; in a somewhat similar sense the response of a nerve or muscle to stimulation is nonspecific; but under some conditions such a response may become specific, as in certain kinds of sensitization (e.g., anaphylactic). Somewhat analogously the starfish egg is nonspecific in its response to parthenogenetic agents, but it is also activated specifically by the sperm of its own species.[8] In the sequence of interlocked physicochemical events which constitute the activation process, it seems necessary to distinguish between certain primary, nonspecific physical events which initiate metabolic reactions of a simple type (e.g., by some kind of surface effect) and later more complex events, following upon the first, into which specific metabolic reactions enter. Such specific reactions may occur at an early stage in fertilization, as seen in the fertilizin reaction of the sea-urchin egg (F. R. Lillie, 1919). The problem of artificial parthenogenesis has reference more particularly to the nature of the initial nonspecific changes, both physical and chemical. The special metabolic and morphogenetic cycle which follows is uniquely characteristic for each species; it may be assumed that each developmental stage has its own special physiological and biochemical features and that the transition to the physiology of the adult organism is gradual rather than abrupt.

On the physical side the nonspecific reaction of activation appears very generally to be associated with a structural alteration of the surface layer or cortical zone of the egg. This is shown with unusual clearness in certain eggs, e.g., the *Nereis* egg (F. R. Lillie, 1912, 1919). The majority of studies have been made with the echinoderm egg; on its first contact the spermatozoon initiates locally a reaction which then spreads rapidly over the whole egg surface (Just, 1922, 1939). One of its manifestations in echinoderm eggs (and some others) is the separation of the fertilization membrane. There appears to be a temporary structural breakdown (of the kind which, if permanent, leads to cytolysis) of the most superficial layer of protoplasm; and this change is, in many cases, associated with an increased permeability to water-soluble substances of low molecular weight and water. This is notably the case in the sea-urchin egg, but in the starfish egg there appears to be no immediate change of permeability on fertilization (R. S. Lillie, 1918, p. 420). In the sea-urchin egg the associated changes of electrical conductivity have been measured by McClendon (1910) and Gray (1913). The connection between the increase of permeability and the metabolic and other immediate effects of activation has been variously interpreted. Substances formerly kept apart by diffusion-proof partitions (such as alveolar walls) may become free to interact when these barriers become permeable, and the resulting reaction may form the initial metabolic step in ac-

[7] Cf. the recent interesting discussion of Dalcq, Pasteels, and Brachet (1936).

[8] Loeb tried to abolish this specificity, or block to fertilization by foreign sperm, and to some extent was able to do so in sea-urchin eggs by making the sea water hyperalkaline (cf. J. Loeb, 1913*a*, p. 225).

tivation. The experiments described above show how the rate of the activation reaction may be influenced by changing the physical density (as determined by water content) of the surface layer of the egg. Other reactions may also be accelerated or enabled to occur by the structural changes of activation. The characteristic metabolic sequence determined by the special organization of the egg may thus be set in motion. Another general physical consequence of increased permeability is a change in the electrical potential difference across the cell surface, and it is possible that such variation of potential (analogous to the action potential of stimulation) may in itself be a critical factor in activation. The facts of electrical parthenogenesis show that the activation reaction may be released by changes of electrical polarization; and the mode of travel of the initial membrane-forming surface reaction (cf. Just, 1922; Whitaker, 1931) suggests its dependence on the formation of local electrical circuits, as in other cases of protoplasmic transmission. A transmitted electrical variation in the frog's egg activated by puncture has recently been demonstrated by Rothschild and Péterfi (1935). Structural changes in the cortical regions of various eggs following activation have been described by many investigators, using a variety of methods. (Some recent papers, giving references to the older literature, are: Runnström, 1928; Hobson, 1932; Chambers, 1933; Moser, 1939; Novikoff, 1939.)

The general view that two processes, physically and chemically distinct, are concerned in activation—(1) a process localized in the surface layer of the egg and associated with a structural change of a cytolytic kind ("lysin theory") and (2) a more internally situated metabolic process involving synthesis—was early supported by Loeb (cf. Loeb, 1913*a*, chap. xii). One of his conclusions was that the critical metabolic change in activation is an increase in certain intracellular oxidations. Increased oxygen consumption in the sea-urchin egg after fertilization was demonstrated by Warburg in 1908 and by Loeb and Wasteneys a little later; and artificial forms of activation (hypertonic sea water, fatty acid) were found to have the same effect.[9] But it has since been found that such a change is by no means general; fertilization may leave respiration unchanged or may even decrease it in some cases, as in the eggs of *Chaetopterus* and *Cumingia*, studied by Whitaker (1933). Even in eggs where fertilization increases respiration, the effect may vary greatly at different temperatures (Gerard and Rubenstein, 1934). Nevertheless, a change in the conditions controlling oxygen consumption appears to be an important feature of activation in certain cases. In the unfertilized sea-urchin egg respiration is low and is almost unaffected by cyanide; but on fertilization it increases several times and cyanide-sensitive factors enter—a fact (among others) indicating that new catalyzing systems come into operation at that time (Runnström, 1930; Korr, 1937; Lindahl, 1938, 1939). A similar increase in respiration can be induced in the unfertilized egg by methylene blue, *p*-phenylene diamine, or pyocyanin (Barron, 1929; Örström, 1932; Runnström, 1930, 1932*a*, 1935*a*), or by nitrophenols (Clowes and Krahl, 1936), but without activating the egg or removing its capacity for fertilization. Increase in respiration is thus not in itself sufficient for activation, although it may be associated with activation as one of its direct or indirect consequences.

Another metabolic effect of activation, which may have a more general importance, is the production of acid; this effect is probably localized in the surface layer of the egg. A temporary release of acid, sufficient to displace considerable quantities of CO_2, was shown by Runnström (1933) to occur in the sea-urchin egg during the period immediate-

[9] Cf. Needham, 1931, 2:623 ff., for an account of this earlier work.

ly following fertilization; and more recent studies by Rothschild (Laser and Rothschild, 1939) indicate that this special acid-producing reaction is completed within a brief period of about 5 minutes. The brevity of this initial reaction is of interest in relation to the fact that in the starfish egg the activating effect of external acid may be completed within an equally short or shorter time. The question as to the nature and source of the acid formed in fertilization is still unanswered; it is also uncertain how far this phenomenon is general and not a special peculiarity of the sea-urchin egg. When we consider the general features of cellular metabolism, it seems natural to refer the acid to carbohydrate metabolism. Fertilization and artificial activation occur readily under anaerobic conditions, as does also the metabolic production of lactic and other acids in many cells. Various attempts have been made, especially by Runnström and his associates (cf. Lindahl's review, 1938), to correlate acid production in eggs with the same phenomenon in yeast, muscle, and other cells. The acid formed in activation is unidentified; lactic acid seems to be excluded by the absence of interference by iodoacetate and fluoride (Runnström, 1935*b* and *c;* R. S. Lillie, 1934); the same conclusion would hold for other acids which arise in the usual types of glycolytic breakdown (e.g., pyruvic), although this may not be certain (cf. Rothschild, 1939). Although lactic acid has been demonstrated in small quantity in both fertilized and unfertilized sea-urchin eggs, there appears to be little change on fertilization (cf. Perlzweig and Barron, 1928). Some rapidly diffusing acid would seem to be indicated by the parallels between heat activation and acid activation in starfish eggs, if we assume that the acid produced in heat activation is the same as in normal fertilization. Heat activation can be arrested at any stage by transferring the eggs to normal sea water at 20°, just as can acid activation. The view that the acid of activation is a lower fatty acid derived from fat metabolism has recently been supported by Hayes (1938), who finds a decrease in the fat content of sea-urchin eggs after fertilization, and he suggests a partial transformation of fat into lower fatty acid.

There are difficulties in regarding acid production as the initiatory metabolic event in all cases of activation. Ammonia is a very efficient activating agent in certain eggs; the *Urechis* egg, recently studied by Tyler, is a good instance (Tyler and Bauer, 1937; Tyler and Horowitz, 1938); Loeb also found various organic bases to be effective in the sea-urchin egg (J. Loeb, 1913*a*, p. 147). In the starfish egg, where the relation of acid to activation is especially clear, ammonia has no activating effect (R. S. Lillie, 1914). Yet the possibility remains that ammonia, because of its special importance in nitrogen metabolism, may form an essential link in the chain of activating reactions. Ammonia penetrates cells readily, and its addition from without might furnish the factor needed to complete activation in some cases. The relation of ammonia metabolism to activation in the sea-urchin egg has recently been investigated by Örström (1937), who finds a marked increase in the assimilation of ammonia after fertilization in the presence of oxygen; this effect is associated with nuclear changes, and he calls attention to possibly significant analogies with ammonia metabolism in the vertebrate liver. In cases where both acid and ammonia may have activating effects, e.g., the *Urechis* egg (Tyler and Bauer, 1937), it would seem that their respective places in the system of activating reactions must be entirely different.

In an earlier paper (R. S. Lillie, 1931), I made the tentative suggestion that a splitting of phosphate esters might form one of the steps in activation, having in mind especially the analogy of muscle metabolism. This would release phosphate from combination; there is, however, no evidence of an increase in free phosphate in the egg during activa-

tion, although this has been looked for (Runnström, 1932*b*; R. S. Lillie, 1934). It should be noted, however, that if the phosphate is not set free within the egg but is simply transferred from one form of ester combination to another, an effect equivalent to acid production might result. The formation of hexose-phosphoric acid, which is a stronger acid than orthophosphoric (cf. Meyerhof, 1930, p. 74; 1937, p. 21), might conceivably be a factor in the observed increase in acidity. This view is consistent with the above described effects of dextrose–sea-water mixtures.[10] A sufficient increase of acidity, in whatever way effected, at the site of the activation reaction (cortical zone) appears to have an activating effect on the starfish egg; in my own comparative study (1927) of the activating efficiency of different acids a constant rate of activation was found to be associated with a nearly constant increase of cH.

Granting the importance of acid, it remains to be determined just how increase of cH can alter the state of the resting egg in such a way as to cause activation. Loeb had suggested as early as 1907 (J. Loeb, 1907) that the primary chemical change underlying membrane formation was some simple splitting process—for example, hydrolysis—and the view that a reaction of this general type may form the first chemical step in activation has been favored by Runnström (1932*b*, 1939) and others. Some compound thus released is regarded as entering directly or indirectly into other reactions of a synthetic kind. Such a conception is in agreement with the general evidence, considered above, that two distinct types of reaction product unite to form a third compound (the "activating substance") the accumulation of which brings the egg into the automatically developing state (R. S. Lillie, 1931, 1934). According to this general view, the activating substance would play a role physiologically analogous to that of a growth-determining hormone.

The problem of the nature of the compound (symbolized as *B* in the scheme) which is progressively released in the egg cortex by increase of acidity is one on which only conjecture is possible at present. Some compound whose diffusion rate is readily affected by changes in the concentration of the protoplasmic gel would seem to be indicated by the experiments with dilute and concentrated sea water described above. Such a molecule must be relatively large; fatty acid diffuses readily into and out of the egg, but the compound formed under its influence is not lost when the egg is transferred to sea water but remains within the system, having apparently entered into some new combination. This is indicated by such facts as partial activation and the cumulative effects of two or more successive treatments with heat or acid. The splitting of a phosphagen such as arginin phosphate would release arginin, the physical properties of which would perhaps answer to the requirements, while as an amino acid this compound might be important in synthesis; but any such identification is purely speculative at present.

The formation of the activating compound *A* (granting it to be formed) represents only a stage or necessary step in the sequence of metabolic events determining development. The initiation of cell division involves changes in both nuclear and cytoplasmic metabolism. Brachet (1938) has demonstrated an increase in thymo-nucleic acid in artificially activated sea-urchin eggs; this increase is similar to that occurring in fertilized eggs but more gradual. There may be a relation here to the changes in nitrogen metabolism described by Örström, since protein metabolism is fundamental in all processes of growth and development. That activation is associated with a change in the physical state of the structural proteins is indicated by the variations observed by Heilbrunn and

[10] However, the phosphorylation of hexoses is inhibited by iodoacetate (as pointed out by Runnström, 1935*b*), which does not prevent activation.

others in the consistency or viscosity of the egg protoplasm following fertilization (Heilbrunn, 1915, 1920, 1937); the increase in free calcium described by Heilbrunn (1937, cf. pp. 538, 544) and Mazia (1937) may have its importance here. Recently Mirsky (1936) has found an increase in the water-soluble proteins of sea-urchin eggs after fertilization.

It is not surprising that the metabolism of artificially activated eggs should agree closely with that of fertilized eggs, since, if activation is complete, both kinds must pass through the same cycle of transformation; and the experiments of Brachet (1938) and others indicate that, as regards oxygen-consumption and other metabolic changes following fertilization, there is no essential difference. Tyler and Horowitz (1938) also find that the initial rate of respiration is the same in the artificially activated as in the fertilized eggs of *Urechis*. As development advances, divergences must be expected, since it is only exceptionally that artificially activated eggs develop without deficiencies of some kind. In this brief review, however, we are concerned only with the nature of the initiating process. The broader problem regarding the nature of the factors which determine and integrate the highly special processes forming the later parts of the developmental sequence is beyond the scope of our present discussion.

SUMMARY

1. Unfertilized starfish eggs, after an exposure to hypertonic sea water of 10–30 minutes, followed by return to normal sea water, show an increased rate of activation by acid (e.g., 0.004 M butyric acid in sea water) or heat (e.g., sea water at 32°). This "sensitizing" effect of hypertonic sea water increases (within certain limits) with concentration and with the duration of exposure.

2. If, however, the eggs are subjected to these activating conditions while they are still in a water-depleted state—for example, if they are transferred directly from the hypertonic sea water to a solution of acid in the same medium—the rate of activation is found to be decreased, to a degree depending directly on the concentration of the hypertonic medium.

3. In these experiments (under 2) the duration of the previous exposure to hypertonic sea water (between 1 minute and 30 minutes) makes little difference in the rate of activation in the acid-containing medium. Since the surface layer (cortical region) of the egg is the only region that can be regarded as reaching a nearly constant water content within so brief a period as one minute, this independence of time is evidence that the site of the activating reaction is superficial, i.e., in the cortical layer of the egg.

4. Similar experiments with dilute sea water (up to 60 volumes per cent) lead to the same conclusion. Temporary exposure of eggs to dilute sea water has, in itself, no activating effect. On the other hand, the immediate influence of dilute sea water on the rate of activation is definite and is opposite in direction to that of hypertonic sea water; i.e., in eggs immersed in dilute sea water, with or without previous exposure to the same medium, the activation reaction is accelerated, to a degree increasing with dilution; this effect is also independent of the duration of the previous exposure.

5. The velocity of the activation reaction thus appears dependent on the water content (structural density) of the cortical region, increasing as the water content increases. This result is interpreted as indicating that the reaction velocity is determined (in part) by the rate of diffusion of reactants in this region, which would appear to have the physical properties of a somewhat dense gel.

6. In mixtures of sea water with isotonic dextrose solution the rate of activation by acid is greater than in normal sea water and increases with the concentration of dextrose (up to 75 volumes per cent dextrose solution).

7. The loss of activability shown normally by starfish eggs kept in sea water beyond the maturation period is delayed or prevented if the eggs are kept in dextrose–sea-water mixtures. (Maturation proceeds normally in such mixtures).

8. A brief general discussion of the physicochemical and metabolic factors of activation is given.

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MITOCHONDRIAL ORIENTATIONS AND MOLECULAR PATTERNS

(Four text figures; one plate)

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THOSE who have studied mitochondria have frequently noted their rather specific orientations in cells where there were no obvious structural features of the cytoplasm, such as cell shape, densely crowded droplets, myofibrillae, etc., to account for such an arrangement. For instance, every textbook of histology states that the mitochondria of columnar epithelium have their long axes parallel with the principal axis of the cell. The author (Pollister, 1932) briefly suggested that these mitochondrial orientations are better described as parallel with the most direct course of diffusion through the cytoplasm. The most conclusive evidence of this is from the large hepatic cells of *Amphiuma* (Text Fig. 1, *A*). The often described orientation "parallel with the main axis of the cell" (Text Fig. 1, *B*) is strictly true only of those parts of the cytoplasm of a columnar cell that happen also to be in the course of diffusion. For above and below the nucleus, which is an obstruction to diffusion, mitochondria are oriented at random. The concentration gradients (Text Fig. 1, *D* and *E*) that determine diffusion through these epithelia are obvious; they are a consequence of the cell's being in a relation where two functional surfaces are in contact with two different fluids, while the other surfaces, the sides, are in contact with adjacent epithelial cells and hence are not functional. In an approximately spherical cell, like a leucocyte floating in blood plasma or tissue fluid (Text Fig. 1, *C*), the entire surface is in contact with the same medium, and there can be no concentration gradients and no diffusion directly across the whole cell. The gradients must, instead, be between the cell surface and the approximate center of the main cytoplasmic mass (Text Fig. 1, *F*); diffusion outward and inward must be along these gradients and hence radial to the approximate center of the main mass of cytoplasm. Mitochondria of the leucocyte are radial to this cytoplasmic center and so, just as in epithelia, parallel with the line of flow or diffusion.

In previous studies (Pollister, 1933*a*, 1933*b*, 1939) it has been noted that the physiological differences between what may be termed the "longitudinally polarized epithelial cell type" and the "centrally polarized leucocyte type" are accompanied by structural differences—in position of centrioles and shape of the Golgi apparatus. In the epithelial cell type the centrioles are at the lumen or distal end of the cell (Text Fig. 2, *A*); in the leucocyte these small granules are near the center of the main mass of cytoplasm (Text Fig. 2, *B*). The epithelial cell has a Golgi apparatus in the form of a vertical collar, so oriented as not to intercept the line of flow of materials through the cell (Text Fig. 2, *A*); in the leucocyte (and other centrally polarized cells, such as connective tissue, myoblasts, and neuroblasts) the Golgi apparatus is a horizontal collar, a perforated disk, likewise oriented to conform to the course of diffusion (Text Fig. 2, *B*).

The present report concludes this survey of the cytoplasmic constituents of the cells of Amphibia with a detailed consideration of orientations of mitochondria.

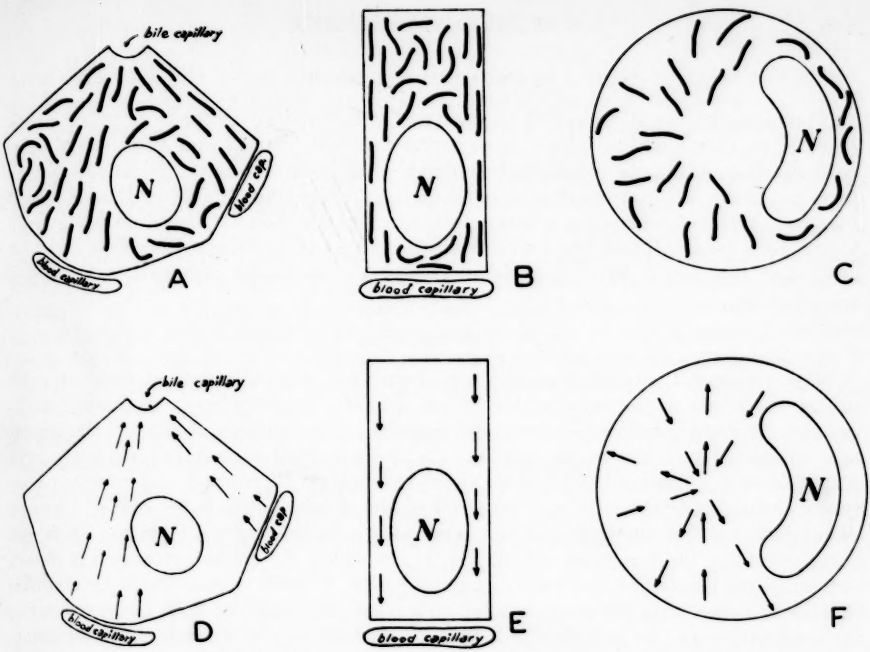

FIG. 1.—Schematic figures of orientations of mitochondria in relation to most direct course of diffusion in tissue cells. Upper row, mitochondria: *A*, hepatic epithelium, *Amphiuma; B*, typical columnar cell. from surface epithelium of intestine; *C*, spherical leucocyte. Lower row, most direct course of diffusion, shown by arrows: between blood capillary and lumen end of epithelial cells, *D, E;* and radial to center of main cytoplasmic mass in leucocyte (*F*), which is in contact with the same fluid over entire surface. *N*, nucleus.

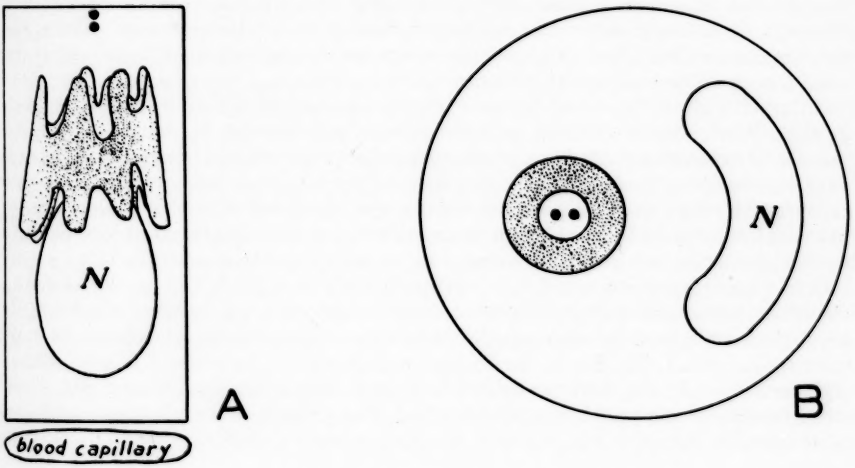

FIG. 2.—Schematic figures of Golgi apparatus, stippled, and centrioles, solid black, in epithelial cell type (*A*) and leucocyte type (*B*). *N*, nucleus.

MATERIAL AND METHODS

The material was all prepared by standard mitochondrial methods of either Benda or of Kull. One must be critical about mitochondrial fixation and must select those cells which contain only rodlike mitochondria of uniform diameter and of nearly uniform length, since the study of cells in tissue culture has made it evident that the granules and shorter filaments are artifacts in most cases, the notable exception being probably all egg cells. All cells are drawn from the tissues of urodelous Amphibia, in which are found the largest vertebrate cells—though by no means necessarily the largest mitochondria.

OBSERVATIONS

Here we are not interested in the mitochondria, per se, but only in their arrangements—as a clue to the organization of the optically structureless protoplasmic background, the hyaloplasm, which must be regarded as the real site of most of the important workaday activities of the cell. In cells that are filled with stored relatively inert material, as in a liver cell (Pl. I, Fig. 2) the hyaloplasm is crowded into interstices between these droplets; and the orientation of the mitochondria is, of course, likewise determined by their being forced into these narrow strands of hyaloplasm. There are other cells, like the fibroblast (Pl. I, Fig. 1), in which the entire cytoplasm is drawn out into long threadlike processes; and, obviously, in such a case the mitochondrial threads can merely be oriented parallel with these processes. In both these cell types the orientation of the mitochondria tells us nothing of the underlying cytoplasmic structure.

There are other cells, notably those of muscle, nerve, and epidermis, where the entire cytoplasmic mass is in a highly organized visible pattern of what are presumably the active functional elements—respectively myofibrillae, neurofibrillae, and tonofibrillae. In these cells the mitochondria are included in this pattern; and the study of their orientation in the fully differentiated cell can teach us nothing additional about the organization of the hyaloplasm. Examination of the mitochondria as these cells differentiate is, however, enormously instructive, for the progress in exact arrangement of these apparently passive rodlets gives us a reflection of the developing molecular organization that results in the differentiated fibrillae (see below, p. 276).

In other tissue cells we see the mitochondria against the typical hyaloplasmic background. When the cell is alive, or when it is as well fixed as it frequently is by the mitochondrial methods, the hyaloplasm appears structureless and homogeneous by ordinary microscopic examination. If there were no mitochondria in it, we should, in most cells, be forced to resort to conjecture as to the organization of this hyaloplasm; or, more likely, we would probably not suspect there was an organization. As it is, mitochondrial orientations give us a definite clue to the cytoplasmic organization.

In a typical leucocyte, a cell that is centrally polarized (Pl. I, Fig. 3), the long mitochondrial threads are oriented at random except where they are radial to a point at the approximate center of the main mass of cytoplasm. There is a similar orientation in the cartilage cell (Pl. I, Fig. 4). In the Champy-fixed material, from which Figures 3 and 4 of Plate I were drawn, there appears to be no structure in the hyaloplasm. But in material fixed in strong protein coagulants (Pl. I, Fig. 5, fixed in Helly's fluid, which contains mercuric chloride) it is clear that the main mass of cytoplasm is organized into an

aster which is radial to the center of the main mass of cytoplasm. As we saw above, this is the point radial to which concentration gradients must be organized.

Figures 6–11 of Plate I are cells of simple, single-layered epithelia. While in all simple epithelia there is a tendency for the filamentous mitochondria that are along the direct path of flow through the cell to be parallel to the path, the accuracy of the parallelism varies with the cell type. The mitochondria are straighter and more nearly parallel in those cells through which the amount of diffusion is greater. Thus the orientation is least marked in the glandular epithelial cells (Pl. I, Figs. 6, 7, 8), where surely there is slow diffusion; it is considerably more marked in the intestinal epithelium covering the villus (Pl. I, Fig. 11), where absorption processes must involve diffusion of a considerable volume of fluid through the epithelial cell; and the parallel orientation is most marked in the cell of the distal segment of the amphibian uriniferous tubule (Pl. I, Fig. 10), which is the part of the tubule through which there is the greatest water transfer (Walker and Hudson, 1937).

Thus, in cells where the cytoplasm consists of mitochondria suspended in hyaloplasm, with no visible complicating cell inclusions or cell organization, the orientation of mitochondria appears to be closely correlated with both the course and the amount of diffusion through the cell. From our consideration of asters, below, we shall see reasons for believing that these mitochondrial orientations are a consequence of the diffusion.

DISCUSSION

A. ORIENTATION OF MITOCHONDRIA

In the differentiated tissue cells we have described above we can only note the correlation between mitochondrial orientations and the course of diffusion. To discover the cause of this orientation it is necessary to examine its development. This has not been done with these tissue cells, but we may understand it from studies that have been made of aster formation in dividing eggs. The reorientations of granules in such eggs are quite comparable with the orientations of mitochondria (which, indeed, some of the granules are [Hibbard, 1922]); and in dividing spermatocytes, which contain only the filamentous mitochondria, these become oriented radially exactly as do the granules of the egg (Terni, 1914; Bowen, 1920; Pollister, 1930). Since it is of easy access, the cytoplasm of marine eggs has been intensively studied. It is densely packed with granules, among which extends the microscopically homogeneous hyaloplasm. The granules may be thrown to one side of the cell by centrifuging, and this reveals the hyaloplasm. As shown especially by the centrifuging experiments of Lillie (1908) and of Conklin (1917), this hyaloplasm, and not the visible granules, is responsible for many of the fundamental and distinctive physical characteristics of the protoplasm. It is responsible for the shape of the cell; it holds the nucleus in position. Within the hyaloplasm—even when it has been completely freed of granules—the asters and spindle of the mitotic apparatus develop and function. The hyaloplasm has a structure or framework which is elastic; and this elasticity is a property which restores cell shape and returns cell constituents to their original positions after their displacement by centrifuging. Orientations within the hyaloplasm (Conklin's spongioplasm) largely determine cell polarity, symmetry, localization of inclusions, etc. The visible inclusions in cells, such as granules and mitochondria, must be thought of as largely suspended in this hyaloplasm; and their movements are probably, for the most part, merely reflections of activity in the

hyaloplasm. The development of an aster, as seen in the normal living eggs, is a good example of this last (Chambers, 1924). It forms in a region that contains densely packed granules. The aster becomes visible in this granular cytoplasm when its rays suddenly develop in the form of clear areas that are entirely free of granules and are radial to a point, the center of the aster. No structure is visible within the rays in the living cell. As one watches the egg, suddenly it is apparent that the granules have become rearranged, so that, whereas before they were densely and uniformly packed, now they are completely absent from certain areas, the astral rays.

From microdissection and centrifuging experiments we know something of the physical nature of the aster. As a whole, the aster is a gel; and it may be pushed about as a single mass by the micrurgist's glass needle. If two asters and a spindle are present, all three together form a highly viscous or gelated region. The centrifuge experiments record this highly viscous state of the aster by showing that it is more difficult to move granules through an egg that contains asters (Fry and Parks, 1934); the viscosity of the egg waxes with the appearance of an aster, and wanes with its disappearance. The astral rays are lines of flow within this gel, for Chambers has shown that fat droplets pushed by the needle into the ray, flow along it toward the astral center.

To return to tissue cells, the aster of the leucocyte must be physically much like the aster of a dividing cell.[1] It appears the same in fixed preparations; and, as we have seen above, radial flow, like the channels called "astral rays" in the dividing eggs, is to be expected from the concentration gradients in the centrally polarized leucocyte; furthermore, the nucleus of the leucocyte is concavo-convex instead of spherical—a fact best explained by assuming that it is molded upon the spherical gelated mass of the aster. We may then conclude that the aster of a leucocyte has arisen by the same sort of organization of the hyaloplasm as occurred in the egg or spermatocyte and that the mitochondria have a radial arrangement because they have been pushed out from the developing rays or channels of flow, just as the granules were moved about in the developing aster of the egg.

Thus the phenomena of aster formation show that, though involving submicroscopic elements in the hyaloplasm, the development of a channel of flow, an astral ray, is carried on with sufficient force to exclude from itself such formed structures of microscopic dimensions as egg granules and mitochondria. Indeed, in the orientation of the latter it seems an especially powerful force, for the mitochondria in the astral region are straightened out parallel with the line of flow (Pl. I, Fig. 3)—a considerable restriction of their normal tendency toward being sinuous bodies. Not only the arrangement but also the shapes of the mitochondria reflect the fact that the hyaloplasm of an aster in a dividing cell, or in a leucocyte, is organized into radial channels of flow in a viscous region.

It is the existence of the channels, or astral rays, that explains the mitochondrial orientation, instead of the reverse. One might, at first, think that the flow occurs in channels because the egg granules or mitochondria cause it to detour, as it were, and that then the more direct channels, involving less detour, would become predominant as the rays. If the channels were thus caused by the presence of the mitochondria, where there are no mitochondria or granules, we should expect no rays but only a sort

[1] There is, however, one important cytological difference: in the dividing cell the aster is formed under the influence of the centriole, while the leucocyte aster is a pure diffusion aster, probably not directly influenced by the centrioles.

of unlocalized general diffusion through the whole region of the aster. But, instead, examination of fixed preparations shows there are well-developed perfectly symmetrical asters in the granule-free areas of a centrifuged egg or in the mitochondria-free regions of spermatocytes, where the precocious nebenkern formation has concentrated all mitochondria in another part of the cell (Wilson and Pollister, 1937). In short, the fact that flow occurs in definitely organized channels must be ascribed to some property of the hyaloplasm itself; it is characteristic of the hyaloplasm to respond to diffusion by the organization of definite channels for flow, which are seen as astral rays in dividing cells and leucocytes.

From this analysis of conditions in the aster it is easy to explain the orientations in the epithelial cell. Here, also, the mitochondria are parallel with the course of flow; and this orientation unquestionably suggests that in the hyaloplasm of the epithelial cell the flow likewise takes place through definite channels, which are functionally strictly comparable with the astral rays. I propose to appropriate the *term* "canaliculi" for these assumed channels in epithelial cells.

The inner-ray material of the aster is, as we have seen, in a highly viscous or gel state. This state of asters and spindle makes nearly the whole dividing cell appear like a highly viscous jelly. It appears from the microdissections of Chambers and Renyi (1925) that the cytoplasm of epithelial cells is likewise, in most cases, a gel. Indeed, since this is known to be true also of nerve cells (Renyi, 1931) and of muscle cells (Speidel, 1938), it seems a very general rule that the cytoplasm of tissue cells is in a gel state.

Let us look at the pictures which, from cytological observations alone, we have thus far painted of the leucocyte and epithelial cells. The cytoplasm consists of hyaloplasm in a gel state and containing suspended visible formed bodies—mitochondria, Golgi apparatus, and centrioles. The mitochondrial orientations especially show us that the hyaloplasm, though apparently unorganized, actually has definite canaliculi, which are the direct channels for diffusion, for they are on a direct line between blood capillary and lumen end of the epithelial cell and on a direct line from cell periphery to center of the spherical leucocyte. Further details of the picture may be best filled in after consideration of the possible molecular basis of the hyaloplasmic structure.

B. STRUCTURE-PROTEINS

Bensley (Bensley and Hoerr, 1934, and Bensley, 1938) has recently given particular attention to the nature of the material that is the basis of cytoplasmic structure, that is, of the hyaloplasm; and he believes it to be due to a special protein fraction, for which I prefer his general term "structure-protein" to the more specific ones he also has suggested.

He extracts from liver cells a protein, which he calls "plasmosin." This very readily forms gels, and upon dilution it is precipitated out as a fibrous mass. From study of fresh tissue and of thin sections of frozen-dried material Bensley concludes that it is "beyond doubt that the plasmosin belongs to the apparently structureless ground-substance [i.e., hyaloplasm, A.W.P.] of the cell, not to membranes, mitochondria, or nuclear chromatin." And Bensley further suggests that since the units of plasmosin confer a certain degree of viscosity on the solution and since they readily collect into long fibers, it seems probable that they are of linear shape.

Very recently these structure-proteins of tissue cells have been briefly reported by

Banga and Szent-Györgyi (1940), who find, as did Bensley, that weak saline extraction removes only a part of the cell protein. The remainder they speak of as "structure-protein." This protein is a definite fraction analogous to myosin, that is, it is composed of rod-shaped molecules, as Bensley believed his plasmosin to be. Upon dilution it forms threadlike precipitates, as do plasmosin and also myosin (Weber, 1934). It is, furthermore, like myosin in the X-ray diffraction pattern and in that, in solution, it gives double refraction of flow.

It thus appears that tissue cells contain a considerable amount of structure-protein, of rod molecules like those of myosin or keratin. As Bensley has shown, this is the basis of structure of the hyaloplasm—or, as Banga and Szent-Györgyi say, it is "the solid edifice of the cell."

Furthermore, Mirsky (1936) has extracted a similar fraction as 12 per cent of the total protein of the unfertilized *Arbacia* egg. "In solution . . . . the protein particles appear to be exceedingly elongated, for the solution possesses high viscosity (a 1.4 per cent solution is 9.6 times as viscous as water), and the relative viscosity decreases when pressure is applied. Furthermore the solution shows double refraction of flow." Mirsky has shown that this protein is intimately involved with the establishment of increased strength and elasticity of the egg cytoplasm that follow fertilization. "The explanation of this increase in strength and elasticity is that in coagulation the elongated protein particles unite to form a fibrous network. This, an early step in the development of the egg, may be regarded as a skeleton framework within which differentiation proceeds."

The structure-protein is like myosin. There is considerable information about the behavior of rodlike molecules in general and about myosin in particular (see, e.g., Edsall, 1938; Mehl, 1938). When in the isotropic unoriented state, i.e., with the principal axes of the molecules free to orient at random, solutions of rodlike proteins form gels, even when very dilute (0.3 per cent in the case of myosin [von Muralt and Edsall, 1930]). These gels, called "thixotropic," have the property of responding to stress by a reorientation of their molecules parallel with the stress lines. Such orientation may be caused by mechanical stress, by stress due to flow, by forces in an electrical field, etc. The larger the molecules the more readily will they orient. In dilute myosin gels the orientation manifests itself in two ways: first, the orientation destroys the isotropic gel state so that the myosin solution will flow; and, second, the oriented molecules doubly refract light between crossed Nicol prisms. Since this latter is readily detected by examining liquid under flow in a polarizer-analyzer apparatus, it is said to be a special property for identification of a thixotropic gel that it shows double refraction of flow. Refraction of flow at relatively low flow stress is also, of course, a practical test for the presence of large rodlike molecules. Solutions of myosin very readily show birefringence due to flow (von Muralt and Edsall, 1930). It is generally assumed that any birefringence in living cells is due to the presence of rodlike elements in a parallel arrangement (see Schmidt, 1937; Seifriz, 1936; Picken, 1940). Where protein with long rod-molecules is known to be present in considerable amount, one may reasonably assume that the birefringence is due to their parallel orientation. Thus the birefringence in parts of a striated muscle fiber is generally ascribed to some symmetrical arrangement of the myosin molecules.

Let us once more get back to tissue cells and dividing eggs. From the work of Bensley, of Banga and Szent-Györgyi, and of Mirsky it is evident that hyaloplasm contains a considerable amount of structure-protein, of long rod-molecules far in excess of that

necessary to form a gel. Since both the leucocyte aster and the hyaloplasm of epithelial cells seem to be a gel (see p. 271, above), it may be tentatively assumed that this gel is due to the presence of the structure-protein and that it is of the thixotopic type,[2] with the rod-molecules oriented at random. The astral rays and epithelial canaliculi, however, are lines of flow, and this flow in a thixotropic gel should cause the rod-molecules of structure-protein to orient parallel to it. Possible molecular orientations in these two cell types are, then, as indicated in Text Figures 3 and 4. This orientation of long molecules parallel with the line of flow we may believe is somehow involved with the exclusion of the mitochondria from the rays and canaliculi.

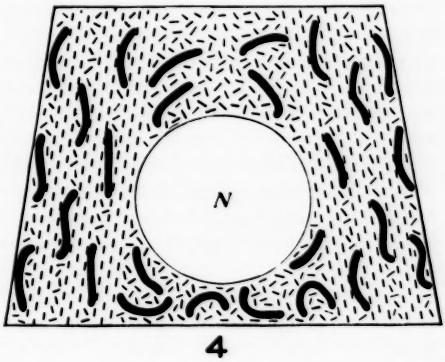

FIG. 3.—Leucocyte cell type. Suggested orientation of mitochondria and rodlike molecules of structure-protein. In the diffusion channels, the astral rays, molecules are oriented by flow and are parallel with the course of flow. It is assumed mitochondria do not enter regions where molecules are thus oriented, but are confined to other parts of the cell where molecules are free to orient at random. *N*, nucleus.

FIG. 4.—Epithelial cell type. Suggested mitochondrial and molecular orientations. Positions of mitochondria, parallel with course of diffusion, suggests that there are definite channels of flow, canaliculi, in which molecules are oriented parallel with the course of flow—as in rays of leucocyte.

The presence of these lines of oriented molecules should be detectable by the birefringence effect; and Schmidt (1936*a*, 1936*b*) has found birefringence in asters of dividing eggs. Schmidt (1937) also cites some evidence of a birefringence in the cytoplasm of epithelial cells, as would be expected if the canaliculi consist of oriented molecules.

The view that the astral rays are lines of oriented molecules of structure-protein explains one apparent discrepancy between the observations on living cells and those on

[2] This assumption of a uniformly unoriented gel outside the rays and canaliculi is, of course, gratuitous. The stresses set up by distortion of a cell fitted into an epithelium must lead to special molecular orientations; and even the almost completely unknown effect of cellular surfaces on orientation may be very significant (see Hardy, 1928, 1932). Indeed, if one considers all these possible orienting forces, it would seem there could be no large intracellular protein molecule completely free to orient itself at random. In the cells we are here concerned with, however, the flow stresses seem to be very significant and to be those most likely responsible for the mitochondrial orientations, just as mechanical stresses surely are in developing muscle fibers (see below, p. 276); and so it seems a justifiable, and possibly more fruitful, simplification to consider the molecular orientations as if they were caused by these forces alone.

fixed preparations. In material fixed by strong protein coagulants the rays are more tenacious of the stain than are the interray regions. If the rays are, as has hitherto seemed most likely, watery channels, this heavier staining is quite unexpected; but it is not surprising if the rays are lines of oriented molecules. Coagulation might then readily cause the molecules to form a stainable, fiber-like artifact.

Since the canaliculi of the epithelial cell appear to be of the same nature as the rays, they also should stain after proper coagulation. They apparently do so, giving the expected picture of stained fiber-like bodies parallel with the line of flow through the cells. This is figured in every textbook of histology, where it is called "basal striation."

C. ORIENTATIONS IN OTHER TISSUES

In the striated muscle fiber it seems certain that the long myosin molecules are, in the mature fiber, arranged in a pattern that is the basis of the visible cross-striations—probably a much more elaborate arrangement than the simple parallel orientation in response to flow that has just been invoked to explain mitochondrial orientations in leucocytes and epithelial cells. As we should expect, the mitochondrial orientations reflect the molecular organization that is developing during the histogenesis of striated Muscle (Lewis and Lewis, 1917, and Friedheim, 1931, on skeletal muscle in tissue culture; Speidel, 1938, on regenerating skeletal muscle *in vivo;* Weed, 1936, on skeletal muscle in fixed preparations; and Goss, 1932, 1933, on cardiac muscle in tissue culture). When a muscle fiber is first capable of contraction, the mitochondria show no particular orientation; there are no myofibrillae or visible cross-striation, and no anisotropy. Molecular orientation is presumably then very slightly developed. As the differentiation proceeds, mitochondria first become parallel with the long axis of the cell. Then, as the cross-striations become visible, the mitochondria are moved about and fitted into this orientation pattern. From his careful observations of living cardiac muscle fibers Goss (1933) says: "We are of the opinion that the mitochondria are imbedded in a contractile matrix or sarcoplasm, and that some mechanical factor is responsible for their rearrangement rather than any direct influence they may have on the contractile power of the cell." It seems likely that, as was assumed for leucocytes, epithelia, and dividing cells, the mechanical factor operating in mitochondrial orientation in the developing muscle fiber is the organization of the myosin molecules into the fibrillar pattern, which must be occurring at this time. From the mitochondrial orientations we may infer that differentiation of the muscle fiber first causes the myosin molecules to assume a fairly simple orientation parallel with the long axis of the fiber. This stage seems to be that observed by the Lewises in muscle buds as a "fine striation which has in general a longitudinal direction," between which the mitochondria are arranged longitudinally. Friedheim has noted birefringence indicative of molecular orientation at this same stage. If the suggestions of Bernal (1938) as to the molecular orientation in muscle are correct, this first orientation may be like that of smooth muscle. Later the myosin molecules assume the much more intricate arrangement that causes the appearance of the alternate isotropic and anisotropic regions characteristic of the fully differentiated cross-striated fiber. In his studies of development of skeletal muscle Friedheim found different physiological properties associated with these two degrees of differentiation, only the second being capable of developing the tetanus characteristic of fully differentiated skeletal muscle. Speidel (1938) has observed that the viscosity is much greater after the cross-striation has appeared in regenerating skeletal muscle. From the experiments of

Weber (1934) with myosin fibers and from analogy with the effect of stretching on cellophane, which is unoriented regeneration cellulose (Seifriz, 1936), it may be concluded that the more nearly parallel molecular orientations in developing muscle confer greater tensile strength on the fiber; and this, in turn, must increase the force of the contraction—and perhaps also its extent. The Lewises (1917), in skeletal muscle, and more recently Goss (1933), in cardiac muscle, have noted that in tissue-cultured muscle the striations first appear in those cells or parts of cells that are subject to mechanical stress due to shape of the fiber and the consequent manner of its attachment to the edges of the clot in which the culture is growing. Goss observed that in the long type of fiber the striations appear nearly simultaneously throughout the whole fiber, with attachment points at either end; while in the irregular fiber, with several attachments, the differentiation occurs first in the areas of greatest stress and later spreads from these centers throughout the whole sarcoplasm. Rodlike molecules like those of myosin have the property of orienting parallel with mechanical stresses, and so what Goss has seen is to be expected. Since the development of cross-striated differentiation leads to increased viscosity and tensile strength and almost certainly to an accompanying increase in force of contraction, in the irregular cell the differentiated areas will serve the more readily to transmit the stresses to the undifferentiated regions and will subject these, in turn, approximately to the stresses that cause orientation of myosin molecules to cross-striated myofibrillae. This will thus involve, finally, the entire fiber.

Observations of the behavior of mitochondria in the differentiation of the neuroblast show that the mitochondria are forced into positions parallel to the neurofibrillae (see especially Hoven, 1910, Figs. 9, 10, 11, 14)—a case that appears to be strictly comparable with muscle histogenesis.

The myoblast may be easily pictured as a system whose structure, with function, must progressively differentiate in the direction of its final stage, the cross-striated muscle fiber. Its anchored ends exert stresses on the cell; each contraction is a momentary increase of the stress, which must certainly cause molecular reorientation parallel with the stress. A residue of each orientation must persist, causing the next contraction to be more powerful; and this increased stress, in turn, will cause more orientation at the next contraction. Eventually we can detect these orientations—first by their effect on the mitochondria, and finally also by the appearance of cross-striation. But the molecular orientation may be assumed to have been thus progressing ever since the first muscle twitch, which may, indeed, have occurred long before the contraction was of sufficient magnitude to be visible.

Thus viewed, the differentiation of cardiac muscle seems a suggestive simple model of histogenesis and tissue determination. If it be assumed, first, that undetermined mesenchyme cells all contain protein that is potentially myosin and, second, that the amount of molecular orientation caused by a few submicroscopic contractions is sufficient to set a mesenchyme cell, if under mechanical stress, on its way to becoming a muscle fiber, then we have a mechanism for determination of this specific tissue.

SUMMARY

The observations show that there are many cells that contain neither stored material nor visible fibrillar differentiations but which, nevertheless, show the mitochondria oriented in a very definite manner. Analysis of this orientation in epithelial and leucocyte types of cells shows that the threadlike mitochondria are parallel to the course of

diffusion in the cell—on a line between capillary and lumen in the former—and radial to the approximate center of the main mass of cytoplasm, which is organized into an aster, in the latter.

It has been shown that the process of formation of astral rays pushes out formed bodies, including mitochondria, from the rays. Furthermore, astral rays are channels of flow. It is concluded, therefore, that the orientation of mitochondria in leucocytes and epithelia is similarly due to their being excluded from special channels of flow—the demonstrated astral rays in the leucocyte, and assumed channels, canaliculi, between basal and lumen ends of epithelial cells.

These orientations are held to be due to the fact that the mitochondria are excluded from regions of rays and canaliculi by the assumption of parallel orientation by long protein molecules—the structure-proteins—of the hyaloplasm. This view finds support in the literature from (*a*) observations of differentiating muscoe and nerve cells, where the mitochondria are similarly forced into positions parallel to the lines of molecules that are becoming oriented into myofibrillae and neurofibrillae; (*b*) the fact that astral rays stain heavily, though known to be channels of flow; and (*c*) evidence of birefringence in asters and in epithelial cells.

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PLATE I

Camera lucida drawings of cells of urodelous Amphibia. In Figs. 6–11 the free (distal or lumen) end of each cell is toward the top of the page; and the lower border of each cell is the basal or proximal end, that adjacent to the blood capillaries.

FIG. 1.—Hepatic epithelium, *Triturus*. In autumn, when cell is filled with stored material in the form of droplets of fat, blackened with osmic acid; and clear spaces, which were formerly occupied by glycogen. Mitochondria restricted to hyaloplasm between these masses of stored material. Kolatchev method. ×2,100.

FIG. 2.—Fibroblast, *Amblystoma opacum* larva. Kull method. ×2,100.

FIG. 3.—Leucocyte, perihepatic zone, *Amphiuma tridactylum*. Small sphere and adjacent black dot are centrosome and centrioles. Kull. ×2,100.

FIG. 4.—Cartilage cell, *Amblystoma opacum* larva. Two small black dots are centrioles. Nucleus not in section. Benda. ×2,100.

FIG. 5.—Leucocyte, like Fig. 3 but fixed in Helly's fluid and stained with iron haematoxylin. ×2,100.

FIG. 6.—Goblet cell, duodenum, *A. opacum* larva. Kull. ×1,200.

FIG. 7.—Pancreas, gland cell, *A. opacum* larva. At upper, lumen, end are prezymogen granules. Kull. ×1,200.

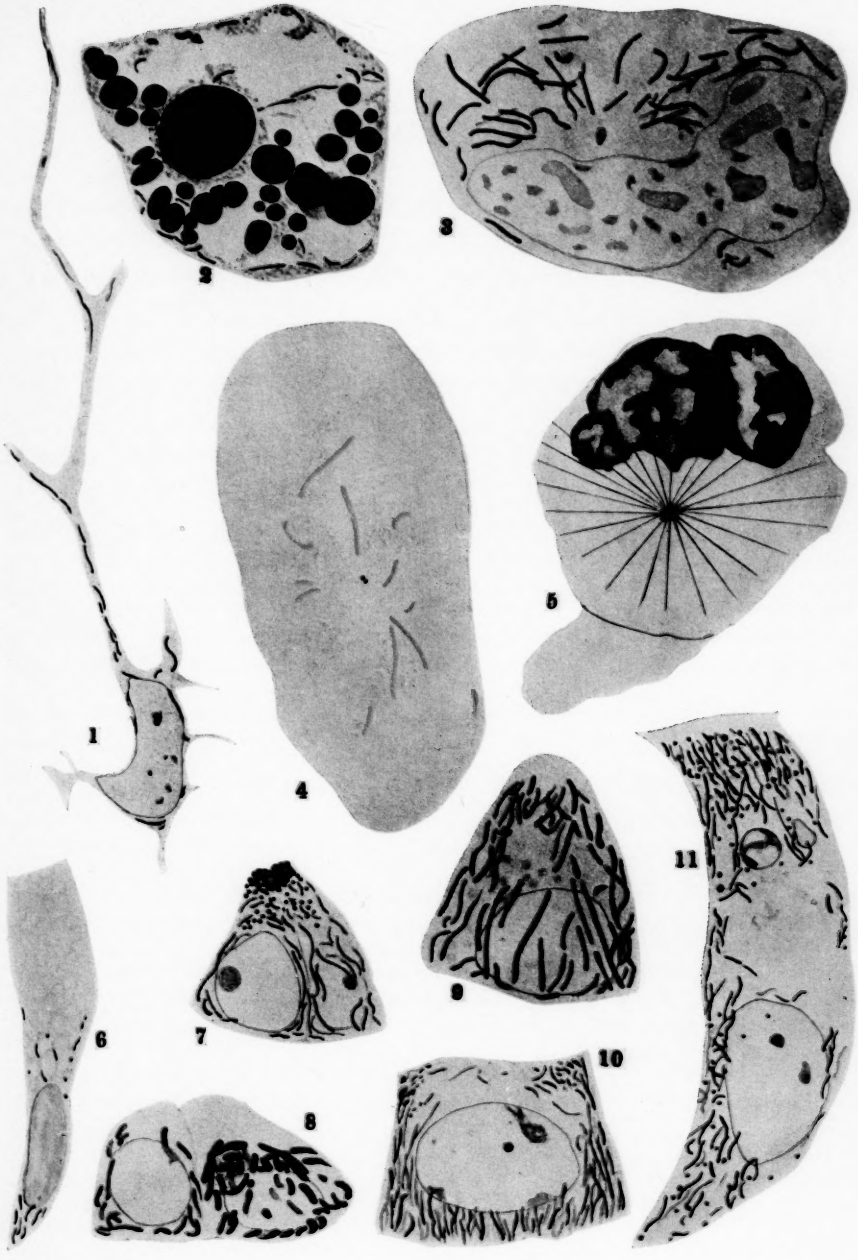
FIG. 8.—Gastric gland cells, *A. opacum* larva. Kull. ×1,200.

FIG. 9.—Epithelial cell, proximal segment of kidney tubule, *Triturus*. Kull. ×2,100.

FIG. 10.—Epithelial cell, distal segment of kidney tubule. *Necturus*. Kull. ×1,200.

FIG. 11.—Epithelial cell, duodenal villus, *Necturus*. Small granules are not mitochondria. Kull. ×2,100.

PLATE I



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THE IRRITABILITY OF FROG MUSCLE IN RELATION TO THE SODIUM ION[1]

(Two figures)

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AMONG physiologists generally, it is commonly believed that the irritability of striated muscle is primarily dependent on the presence of sodium ion. This belief is based on a remarkable paper published thirty-nine years ago by Overton and never, to the best of our knowledge, seriously challenged. Overton's work is so impressive that it is easy to understand why it has had such a lasting influence. Standard books in the field (e.g., Höber, 1926; Lillie, 1932) provide extensive summaries of Overton's experiments, and they accept his conclusions without question.

What Overton did was essentially this: Frog muscles were suspended in approximately isotonic solutions of sucrose and other sugars. In such solutions they lost their power to respond to direct stimulation with an induction coil. Small muscles, such as the sartorius, became nonirritable in a relatively short time (in as little as an hour or less); however, the gastrocnemius required somewhat longer immersion before its irritability was lost. Following loss of irritability, when the muscles were returned from sugar solutions to solutions of sodium chloride or other sodium salts, direct excitability was regained. More than any other cation, sodium was effective in restoring the irritability lost in sugar solutions; lithium was also effective; but calcium was relatively without effect. Indeed, in his 1902 paper Overton stated that irritability could not be restored by calcium, but later (1904) he retracted this statement and admitted that irritability lost in sugar solutions could, to some extent, be restored by calcium if the proper concentration of calcium was correctly hit ("passend getroffen").

In his first attempt to explain the action of sugar Overton decided that sugar acted by replacing with a nonconducting solution the electrolyte medium which normally surrounds the muscle fiber, thus rendering electric stimulation ineffective because of failure of the stimulating current to reach the tissue. He was so sure of this interpretation that he called in his teacher (von Frey) to witness a proof of the hypothesis. A nerve-muscle preparation was rendered nonirritable by immersion in a sugar solution; then the nerve alone was removed from the sugar solution, immersed in a sodium chloride solution, and stimulated electrically. Overton apparently expected the nerve to regain its irritability as soon as the sodium chloride was able to conduct the electric current to the nerve fibers; and he expected, further, that if the nerve fibers were aroused the muscle would, of necessity, contract. The fact that the muscle did not contract indicated to Overton that his (original) hypothesis was incorrect, and he concluded that the sugar must render the individual muscle fibers nonirritable. Further evidence in support of this view came from the observation that the nerve to the gastrocnemius muscle did not lose its irritability even after long immersion in sugar solution. It is, of course, obvious that loss of indirect excitability of a nerve-muscle preparation may be due to loss of irritability of the nerve,

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the muscle, or the myoneural junction; but, strangely enough, throughout his 1902 paper Overton never considers the possibility that loss of indirect excitability may depend, in part at least, on the myoneural junction. Later, in 1904, Overton does discuss the myoneural junction at considerable length, but by this time he had completely abandoned his original hypothesis.

Actually there are two separate questions to be answered. In the first place, is the direct excitability of the muscle cells themselves primarily dependent on sodium ion? And, secondly, is the indirect excitability of muscle—that is to say, its response to stimulation through its nerve—primarily dependent on sodium ion?

To the first of these questions Overton's answer was simple. He held that in sugar solutions muscle fibers lose their irritability because sodium ion is removed from the tissue spaces which constitute the immediate environment of the fibers, and that replacement of sodium results in a restoration of irritability.

Concerning the indirect excitability of muscle, there can now be no question. It is primarily dependent on calcium ion rather than on sodium ion. Indeed, in sodium chloride solutions indirect excitability is lost and can only be recovered when calcium ion is added. This is indicated by the early work of Locke (1894) and Cushing (1901). Overton was unaware of this early work when he published his 1902 paper, but he knew of it by 1904 and was able to confirm it. Since then it has received frequent additional confirmation (see Poliakoff, 1904; Joseph and Meltzer, 1911; Kleefeld, 1913; Taubmann and Hikiji, 1931; Lapicque and Lapicque, 1933; Taubmann, 1934; Feng, 1936; Chao, 1936, 1937; Cicardo, 1938). Moreover, in experiments of our own on sartorius and semitendinosus muscles we have obtained convincing proof that calcium ion must be present if indirect excitability is to be maintained. Muscles which have lost their indirect excitability following immersion in sugar solution for 20–30 minutes recover it only when returned to solutions containing calcium ion. In view of the general agreement concerning the need of calcium ion for indirect excitability it is scarcely necessary for us to submit a complete description of these experiments, and we shall confine our attention to the question of the relation of direct muscle excitability to the presence of sodium ion.

CORRECTNESS OF OVERTON'S BASIC EXPERIMENT

It is a simple matter to show that, if a frog muscle is immersed in an isotonic solution of sucrose, after a time it gives no obvious response when stimulated directly with an induction coil; it is also easy to show that, following transfer from the sugar solution to a solution of sodium chloride, the same electric shocks which were previously without obvious effect now produce well-marked contractions of the muscle. Observations of this sort have been made not only by Overton but also by various other authors, including Schwartz (1907), Höber (1907), and Chao (1937). They can readily be confirmed, and from the outset we realized that Overton's basic experiment was undoubtedly correct in so far as the facts go.

Although Overton reported his experiments in detail, his observations on the length of time necessary for loss of irritability are not always clear. Thus, in some instances he stated that a muscle was nonirritable at a certain time, without giving any assurance that this particular time was the exact moment or even the approximate moment at which irritability was lost. For sartorius muscles in sucrose solutions Overton (1902) notes the following times for loss of direct excitability: 45, 30, and 120 minutes. In one experiment with glucose solution a sartorius muscle lost its irritability in 40 minutes.

These are the only observations recorded in the 1902 paper for sartorius muscle. Loss of irritability occurred more rapidly in the muscles of the toes, especially the smaller toe muscles. Gastrocnemius muscles took longer times to lose their irritability. In the 1904 paper the times required for loss of irritability in sugar solutions were longer, with the exception of one case (Expt. 13) in which apparently only an approximate time was given. The actual data are 60, 300, 114, 258, 182, 140, 87, 102, and 147 minutes for sartorius muscle. Some of the longer times are certainly due to infrequency of observation, but in one case (Expt. 22) Overton states that the muscle was still irritable after 115 minutes' immersion. It is thus incorrect to say, as do Fenn (1931) and Victor (1933), that Overton showed loss of irritability of muscles in sugar solutions in 15 minutes.

As a matter of fact, the time required for a muscle immersed in sugar to lose its irritability is largely a function of the method of observation. When a muscle is first placed in a sugar solution, it undergoes violent twitching and contracture. After the twitching ceases, if the muscle is then tested by any sort of electric stimulus, its reponse is decidedly less than that which a similar shock would have produced if the muscle were in Ringer's solution or in a solution of sodium chloride. If the shocks are repeated at intervals of several minutes, before long there is only a localized response, and this gradually becomes less and less visible. It is often difficult to determine exactly when the last traces of response disappear. In our experiments we observed the response of the muscle with a low-power microscope, and under such conditions the times required for complete loss of response are typically higher than any recorded by Overton. The last traces of irritability can generally be observed only in a few fibers in the vicinity of the electrodes. Moreover, the region of the muscle around the anode has a higher threshold than the region around the cathode.

IRRITABILITY OF INDIVIDUAL FIBERS AFTER PROLONGED IMMERSION OF MUSCLES IN SUGAR SOLUTION

The fact that in sugar solution the response of a muscle may become extremely localized leads to the suspicion that there may be an element of truth in Overton's original hypothesis. The fibers close to the electrodes respond to an electric shock presumably not because they are more sensitive than other fibers of the muscle but because the electric current reaches them and does not reach more remote fibers. This is not the complete explanation of the effect of the sugar solution, for an additional factor is almost certainly the failure of muscle fibers themselves to conduct the response to stimulation from one region of the fiber to another.

If Overton's original hypothesis is in part correct, then after muscles have been immersed for long times in sugar solutions it should be possible to dissect from such muscles fibers still sensitive to electric stimulation. This is really a very simple experiment to perform. After muscles have been in sugar solutions for a long time, fibers are teased out from them with ordinary dissecting needles, and the irritability of these individual fibers can then be tested. Experiments of this sort are reported in Tables 2 and 3, which appear in a later section of this paper. The experiments of Table 3 are the more convincing. In 13 experiments of this table sartorius and adductor magnus muscles were immersed in isotonic solutions of sucrose for longer than an hour, and yet in every case fibers isolated from the muscles proved irritable. In 2 cases the individual fibers were irritable, even though the muscles from which they had been taken had spent over 4 hours in the sugar solution.

In Tables 2 and 3 the thresholds of the whole muscle in sugar solution are also given, and it is to be noted that irritability persists for long times. However, as previously stated, these determinations were made under a microscope, and the threshold voltages concern only the contraction of very few fibers close to the electrodes. It is thus apparent that when muscles are immersed in sugar solutions individual fibers retain their irritability for much longer times than Overton supposed.

The conclusion to be derived from such observations seems inescapable. In Overton's experiments failure of sartorius muscles to respond to stimulation after immersion in sugar solutions for an hour or two may well have been due largely to the fact that the applied electric current did not reach individual fibers remote from the electrodes.

IF OVERTON'S ORIGINAL HYPOTHESIS IS PARTLY CORRECT, THEN WHY DOES NOT ANY ELECTROLYTE ACT LIKE SODIUM CHLORIDE?

Clearly, the transfer of muscle from sugar solution to a solution of any electrolyte should provide a good conducting medium in and around the individual fibers of the muscle. Accordingly, if Overton's original hypothesis is correct, transfer from sugar solution to any electrolyte solution should quickly restore response to electric stimulation. Overton's experiments indicated that this was not the case. And yet these experiments do not constitute a disproof of his original hypothesis, according to which response fails to occur in a nonelectrolyte because of the failure of the stimulus to reach the tissue. The reason that sodium chloride is distinctive in restoring irritability may lie in the fact that sodium is relatively nontoxic. If a muscle, after having lost its irritability in sugar solution, is transferred to a solution of potassium chloride, one could scarcely expect the irritability to return, for potassium itself can rob a muscle of irritability. According to Höber (1926), this has been known since the time of Claude Bernard.

On the other hand, if a muscle is transferred from a sugar solution to a solution of calcium chloride, pronounced shortening occurs. The shortening might tend to prevent additional contraction on stimulation by an electric current. If, instead of transfer to isotonic calcium chloride solution, transfer is made from the sucrose solution to a solution of sucrose plus a small percentage of isotonic calcium chloride solution, then, if Overton's original hypothesis were correct, one might expect that under favorable conditions there would be at least a transitory return of irritability. This is exactly what does occur, as Overton himself found (see Overton, 1904, p. 222) and as we have confirmed. See also Kleefeld (1913).

Although calcium in proper concentration can cause a return of muscle irritability following immersion in sugar solution, this return is never as complete as that obtained in solutions of sodium salts. Later we shall cite experiments to show that, for the isolated single fiber, calcium is far more effective than sodium in restoring irritability following immersion in sugar solutions. Indeed, sodium is not effective at all. It is thus evident that the behavior of the single fiber is different from that of the whole muscle. The reason for this difference is not wholly clear. It is obvious, however, that the entire muscle is a complex system in which various secondary effects may be produced. We believe, therefore, that experiments on single fibers yield results of greater significance than those obtained on the whole muscle.

THE RELATION OF THE SODIUM ION TO RESPONSE TO MECHANICAL STIMULATION

In a sentence or two at the end of his 1902 paper Overton cites two arguments against his original hypothesis. Loss of irritability in sugar solutions, he states, cannot be due to

increase of electrical resistance, for the muscle in sugar solution is also nonirritable to mechanical stimuli. And, second, irritability to electric stimuli is only very slightly lowered in mixtures of sugar and sodium chloride solutions, and not until the salt concentration falls below 0.12 per cent does a sharp drop in irritability occur.

The second of these arguments does not seem very important to us. The relation of a muscle to a stimulating electric current is a complicated one. With decrease in the conductivity of the solution in which the muscle and its fibers are bathed, there is a decrease in the total amount of current passing through the system; but, at the same time, with decrease in the conductivity of the fluid surrounding the fibers, a greater proportion of the current is shunted through the fibers and less of it passes between the fibers. Thus, as salt concentration is lowered, the first factor tends to decrease the effect of an electric current, whereas the second factor tends to increase it. With a system of two opposing factors such as these, it is hard to know just what is happening. Certainly it is quite conceivable that a sharp drop of irritability when the salt concentration falls below a certain point may be due to purely electrical factors.

On the other hand, Overton's first point is an important one, in spite of the fact that it is based only on two recorded experiments (Nos. 10 and 11). In these experiments Overton's primary concern was in another direction. Nevertheless, he did show that, following immersion in sugar solution, muscles failed to respond to the mechanical stimulation resulting from ligature with a silk thread. Moreover, on return from sugar solution to a solution containing sodium chloride, irritability to mechanical stimulation (by ligature) was recovered.

It can readily be shown that Overton was correct in stating that irritability to mechanical stimulation is lost in sugar solution and recovered in solutions of sodium chloride. Moreover, such irritability is not recovered in solutions of potassium or calcium chloride, for experiments of our own have demonstrated this fact.

A point which Overton did not recognize is that irritability to mechanical stimulation is lost much more rapidly than irritability to electrical stimulation. This is shown by a series of experiments in which muscles, on being placed in sucrose solution (0.234 molal), were stimulated at short intervals by being struck a sharp blow with the edge of an ordinary microscope slide. This type of mechanical stimulation is easier to apply than a ligature and apparently produces less injury. In Ringer's solution, when a sartorius muscle is struck while it is being observed under the microscope, it is easy to see that fibers in the neighborhood of the struck area show irregular and periodic twitching. There may also be some contracture localized in the immediate neighborhood of the blow. Table 1 shows the time necessary for loss of response to mechanical stimulation following immersion in isotonic sucrose solutions.

It is clear that loss of irritability to mechanical stimulation occurs very rapidly in sucrose solutions—far more rapidly than loss of irritability to electric stimulation. Perhaps this means little more than that mechanical stimulation is not as powerful as strong electrical stimulation, but to us such an interpretation seems somewhat doubtful.

Certainly, although Overton reported only 2 experiments with mechanical stimulation, his case for the necessity of sodium salts seems much stronger for this type of stimulation than it is for electrical stimulation. It would be interesting to discover if the individual fibers of a muscle immersed in sugar solution retained irritability to mechanical stimulation. Such an experiment is not easy to perform, since in our experience any mechanical stimulus strong enough to evoke a response of any sort, usually a localized contracture, also caused a visible injury. This injury appeared to be a localized, largely

irreversible shortening, closely resembling the plug formed at the cut ends of a fiber in the presence of calcium (Heilbrunn, 1940).

It might be possible, therefore, to assume that mechanical stimulation produces slight local injuries and that these injuries in themselves are the direct cause of the response. Immersion in sugar, with consequent removal of calcium ion, should therefore prevent response to mechanical stimulation, for it is the calcium ion which is primarily responsible for the plug reaction. Against such an interpretation is the fact, readily observable, that a muscle which has lost its irritability to mechanical stimulation by immersion in sugar solution regains its irritability on being placed in a solution of pure sodium chloride. However, it is possible that when the muscle is placed in the sodium chloride solu-

TABLE 1

MECHANICAL STIMULATION

A				B			
Time for Loss of Irritability in Sucrose* (Minutes)	Duration of Immersion in Sucrose (Minutes)	Recovery Medium	Time for Return of Irritability (Minutes)	Time for Loss of Irritability in Sucrose (Minutes)	Duration of Immersion in Sucrose (Minutes)	Recovery Medium	Time for Return of Irritability (Minutes)
sa. 7	20	NaCl	8	7	20	Na oxal.	7
st. 7	20	NaCl	7	17	20	Na oxal.	no recovery
st. 6	10	NaCl	4.5	6	10	Na cit.	16
il. 6.5	10	NaCl	5	6.5	10	Na cit.	no recovery
sa. 5	64	NaCl	8	5	64	Na cit.	no recovery
il. 7	62	NaCl	9	7	61	Na cit.	no recovery
sa. 10	12	NaCl	2.5	12	13	Na cit.	2.5
st. 12	13.5	NaCl	6.5	14	15	Na cit.	no recovery
sa. 29	29	$NaNO_3$	20	30	30	Na cit.	no recovery
sa. 19.5	20.5	$NaNO_3$	4	21.5	21.5	Na cit.	no recovery
st. 18	19	$NaNO_3$	3	20	20	Na cit.	no recovery
st. 17.5	17.5	$NaNO_3$	11	19	19.5	Na cit.	no recovery

* sa. = sartorius; st. = semitendinosus; il. = ileofibularis.

tion, after the brief immersion in sucrose necessary for loss of mechanical irritability, some calcium is still available within the muscle and is released. This notion of calcium release by sodium solutions was evoked by earlier students of ion physiology to explain some of the puzzling phenomena they encountered (see, e.g., Martin, 1904, 1906). Recently, there has been some direct evidence to show that bound calcium in cells is actually set free by pure sodium chloride solutions (Mazia, 1933), just as in blood, calcium is set free from calcium proteinate by sodium ion (Loeb, 1924; Loeb and Nichols, 1927).

There is also a possible explanation in terms of electrical theory. If mechanical stimulation must cause injury to produce an effect, then the response may be essentially due to the electric currents which arise as a result of the injury. Such currents would cease to flow when the muscle is immersed in a nonconducting sugar solution but would be restored on return to a sodium chloride solution. This type of interpretation would obviously be an extension of Overton's original hypothesis. Injury currents in muscle have voltages of 50 millivolts or more, and such voltages may be enough to cause response,

especially in cases in which mechanical impact causes disturbances in position of the fibers, so that several injured fibers might have their currents arranged in series.

Although it is possible that mechanical stimulation, to be effective, must involve localized injuries of individual muscle fibers, this is not a thesis we should like to uphold as constituting a complete explanation of the facts. There is another type of interpretation which is perhaps more important. In the first place, it should be recognized that sodium ion and mechanical shock both have similar effects on muscle. It has long been known that a muscle immersed in a pure solution of sodium chloride will respond by twitching. In such a response individual fibers of the muscle contract independently of each other to give the characteristic appearance of fibrillation well known to muscle physiologists. Following mechanical stimulation, the response is, to some extent, similar. Moreover, muscles which have lost irritability to mechanical stimulation by brief immersion in sugar solution, on being placed in sodium chloride solutions begin to show spontaneous twitchings at about the time they regain their irritability to mechanical stimulation. In such muscles placed in sodium chloride solutions following loss of irritability in sugar solutions, it can be assumed that the sodium ion acts synergistically with the mechanical stimulation. In other words, the twitchings which tend to be initiated by the sodium ion alone are enhanced by mechanical stimulation, and vice versa (compare Adrian and Gelfan, 1933). Now it has been shown that the response of an isolated muscle fiber to sodium ion depends on the presence of calcium ion in the surface or cortex of the fiber (Ashkenaz, 1941). It seems likely, therefore, that response to mechanical stimulation is also dependent on the presence of calcium ion in the cell cortex.

We have performed one experiment which favors this interpretation. If, as Overton believes, it is the sodium ion alone that is essential for the contraction of muscle, then it should be immaterial which sodium salt is added to restore irritability. However, if muscles which have lost their irritability to mechanical stimulation by brief immersion in sugar solutions are placed in isotonic solutions of various sodium salts (chloride, nitrate, oxalate, citrate), then the chloride and nitrate restore irritability to mechanical stimulation in a few minutes, whereas the citrate and oxalate, which tend to remove active calcium ion from solution, do not generally favor restoration of irritability. This is shown in Table 1, which represents experiments on matched pairs of muscles. The muscles in the A group were exposed first to isotonic sucrose solution and then to an isotonic solution of either sodium chloride or sodium nitrate. The corresponding muscles of the B group were transferred from the sugar solution to isotonic solutions of sodium oxalate or sodium citrate. In all 12 experiments, return to sodium chloride or sodium nitrate resulted in a restoration of irritability to mechanical stimulation. On the other hand, in 9 out of the 12 cases no restoration of irritability occurred when the muscles were transferred from the sugar solution to the oxalate or citrate solution.

These experiments on mechanical stimulation lead to no very definite conclusions as to the need for sodium ion in the response of the muscle fibers to mechanical shock. For one thing, we have not been able to exclude the possibility that mechanical stimulation may depend on injury. It is true that solutions of sodium chloride restore irritability to mechanical stimulation after this has been lost by immersion in sugar solutions. It is possible that the restorative effect of the sodium chloride solution may be due to a release of calcium ion to the intercellular spaces of the muscle. It is possible, also, that the sodium chloride may cause a release of calcium ion within the substance of the muscle fibers themselves. In support of such a possibility is the fact that the stimulating action of

sodium ion on cells can generally be interpreted as being due to a release of calcium to the cell interior, as a result of the action of the sodium ion on bound calcium present in the cell cortex (see Heilbrunn, 1937, p. 378, 438; also Heilbrunn and Wilbur, 1937). If, following brief immersion of muscles in sugar solution, transfer to sodium chloride solution did cause a release of calcium ions to the interior of the muscle cells, then the sodium ion might be thought of as acting synergistically with mechanical stimulation; and, indeed, sodium ions and mechanical stimulation produce somewhat similar effects on muscle. The fact that sodium oxalate and sodium citrate typically do not restore irritability to mechanical stimulation is an indication that the calcium ion may be necessary—if not in the medium, at least in the fiber itself. Further study will eventually clarify the situation. For the present, one cannot state with any assurance what the role of the sodium ion may be in mechanical stimulation. As will be noted later, the results for electrical stimulation are much clearer.

THE RELATION OF THE SODIUM ION TO THE RESPONSE OF ISOLATED MUSCLE FIBERS TO ELECTRICAL STIMULATION

In an earlier section it was noted that, when frog muscles were immersed in isotonic sucrose solution for times sufficient to insure loss of visible response to stimulation by induction shock or condenser discharge, that, following such loss of irritability, the individual fibers, on being dissected from the muscle, usually responded to electrical stimulation even though still immersed in sucrose solution. Now the question arises as to the effect of sodium ion on the irritability of such isolated fibers. If Overton's major point is correct, then sodium ion—and sodium ion alone—should favor the response of these fibers to electrical stimulation. This is perhaps the crux of the whole problem. We shall present evidence to show: first, that the excitability of the isolated muscle-fiber preparation is favored by calcium ion alone; second, that sodium ion decreases the excitability; and, third, that the depressing effect of sodium on excitability can be neutralized by the addition of calcium ion.

First as to method. Immediately after a frog was pithed, the muscles desired for study were placed either in an isotonic sucrose solution (0.234 molal) or in Ringer's solution. The muscles used were sartorius and adductor magnus. The Ringer's solution consisted of the following: 0.65 gm. NaCl, 0.02 gm. KCl, 0.026 gm. $CaCl_2 \cdot 2H_2O$, distilled water to 100 cc. This was buffered by adding M/15 Na_2HPO_4 to pH 7.3–7.5. The concentration of the sodium chloride solution used was 0.73 per cent (approximately M/8). Such a solution is isosmotic with frog blood (Hill and Kupalov, 1930). Calcium chloride was used in 0.09 M solution. This is isosmotic with the sodium chloride solution, as determined by calculation from freezing-point data.

After a little practice isolated muscle fibers are easy to obtain, especially from the adductor magnus muscle. The individual fibers are teased away from their neighboring fibers by the use of ordinary dissecting needles. Then a convenient length of the fiber is cut and is ready for study. As a result of the method of preparation the fiber is cut at both ends and is therefore not wholly normal. Indeed, from the moment of preparation it begins to die from both cut ends. Certainly a superior preparation from the standpoint of normality is that of Ramsey and Street (1938). However, the Ramsey and Street preparation takes far longer to prepare and is therefore not so convenient for extended series of experiments. If care is taken to avoid excessive injury, the type of preparation

we employed may retain its viability and its irritability for several hours. Soon after isolation, however, the cut muscle fiber loses the power to transmit a stimulus from one region to another (cf. Brown and Sichel, 1936). In other words, the fiber usually responds only at the point at which it is stimulated. Our data are, therefore, concerned mainly with this type of response and should accordingly be interpreted from this standpoint.

In order to obtain a quantitative measure of the irritability or excitability of the individual fibers, their threshold to electric stimulation was determined. In every case the threshold voltage was that voltage to which a 4-microfarad condenser had to be charged and which, when discharged through the electrodes, would produce the smallest observable response in the fiber. The electrodes were fine silver wires, coated with AgCl and kept at a distance of 10 mm. from each other. They were not allowed to touch the fiber; but an attempt was made to keep the same relation between electrodes and fiber in any given experiment, in order to minimize the effect on the threshold of varying the resistance between the electrodes.

In our measurements, obviously, it is not possible to determine what proportion of the stimulating current passes through, and what portion passes around, the fiber. This is a difficulty that is attendant on practically all measurements of threshold to electric stimulation. It becomes particularly serious if one attempts to compare threshold measurements made in solutions of widely different conductivities. Thus, for example, the threshold of a muscle fiber immersed in sugar solution may be greater or less than that of the fiber immersed in isotonic salt solution, and yet this need not necessarily mean that the fiber is less or more irritable. In interpreting our data, therefore, no attempt should be made to regard the values for thresholds as having an importance as absolute values. Nevertheless, with reasonable caution they can certainly be used for purposes of comparison; and, used in this way, they provide ample proof for our claim that it is the calcium ion, rather than the sodium ion, that is essential for the irritability of the fiber.

Table 2 shows the results of experiments in which fibers were dissected out from mus, cles previously immersed for long periods in isotonic sucrose solution. In the first column the numbers in parentheses identify the individual muscles. Following isolationsome of these fibers were still irritable to the stimulation we had available, although others were not, as indicated by the fact that their threshold is given as being above 105 volts. When these fibers were transferred to isotonic calcium chloride solutions, all but 2 of the fibers showed a marked decrease in threshold. In these 2 instances the fibers did not recover their irritability. Immersion in the calcium chloride solutions was brief, for the fibers live only a short time in such solutions (Heilbrunn, 1940).

It might be suspected that the marked drop in threshold on transfer to the calcium chloride solution was due to the increased conductivity of the salt solution. If this were the case, then transfer from sugar solution to solutions of sodium chloride should have a similar effect. But exactly the opposite is true. This is shown by Table 3, in which, as before, the number in parentheses in the first column identifies the individual muscles. In the experiments listed in this table, of 14 cases in which fibers were dissected out of muscles immersed for long times in isotonic sucrose solution, in nearly every instance the fibers were found to be irritable. But when these irritable fibers were exposed even for a brief time to isotonic solutions of sodium chloride, all but 2 of the fibers showed a complete loss of irritability. Following this loss of irritability (in 10 out of 12 fibers), a small amount of isotonic calcium chloride solution was added to the drop of sodium chloride solution in which the fibers were bathed. As a result, there was a sharp drop of threshold

TABLE 2

Fibers Dissected from—	Duration of Immersion of Muscle in Sucrose	Threshold of Muscle before Dissection of Fibers (Volts)	Threshold of Fibers on Isolation (Volts)	Duration of Immersion in $CaCl_2$ (Minutes)	Threshold in $CaCl_2$ (Volts)
Sartorius (2)	1 hr. 15 min.	66	75	1	36
	1 24	66	75	1	24
	1 24	66	45	1	24
	1 45	66	105	0.25	9
Sartorius (3)	0 37	3	105	4.5	15
Sartorius (4)*	0 54	>105	60	1	2
	2 28	>105	>105	0.5	>105
Sartorius (7)	2 33	6	>105	0.25	102
	2 49	6	>105	0.5	>105
	2 54	6	36	2.75	18
	3 18	6	>105	3.5	30
	3 51	6	>105	1	60

* The behavior of this particular fiber is shown graphically and in more detail in Fig. 1.

TABLE 3

Fibers Dissected from—	Duration of Immersion of Muscle in Sucrose	Threshold of Muscle before Dissection of Fibers (Volts)	Threshold of Fibers on Isolation (Volts)	Duration of Immersion in NaCl (Minutes)	Threshold in NaCl (Volts)	Duration of Immersion in NaCl+ $CaCl_2$ (Minutes)	Threshold in NaCl+ $CaCl_2$ (Volts)
Adductor magnus	2 hrs. 12 min.	.....	2	3	45	2	30
Sartorius (1)	3 16	.....	39	1	>105	1	>105
Sartorius (2)	1 06	66	45	1	>105	0.5	30
	1 36	66	75	0.5	>105	1	18
	1 41	66	18	0.5	>105	0.5	45
	1 47	66	39	1	>105	0.75	49
Sartorius (3)	0 33	3	36	0.25	>105	0.5	60
Sartorius (4)	2 24	>105	84	0.5	>105	0.5	96
Sartorius (5)*	3 01	>105	15	0.25	>105	0.75	45
Sartorius (6)	4 30	.....	105	0.5	>105	1	>105
	4 39	.....	96	1	93	0.5	45
Sartorius (7)	3 01	6	105	0.5	>105	4.5	39
Sartorius (8)	2 23	30	105	0.25	>105	0.25	69
	2 26	30	2	1	>105	2.5	10

* The behavior of this particular fiber is shown graphically and in more detail in Fig. 1.

in all but 2 of the preparations. (In these 2 there was no recovery of irritability.) In the case of these fibers dissected out of muscles previously immersed for a long time in sucrose solution, the results show clearly that immersion in sodium chloride, instead of favoring a restoration of irritability, as Overton's views would lead us to believe, actually reduces or destroys irritability. Clearly, it is the calcium ion which is important for the response of these isolated fibers to electrical stimulation. The data presented in tabular form do not give a complete record of changes in threshold in relation to time as well as to changes in medium. Graphic records could be presented for each of the experiments

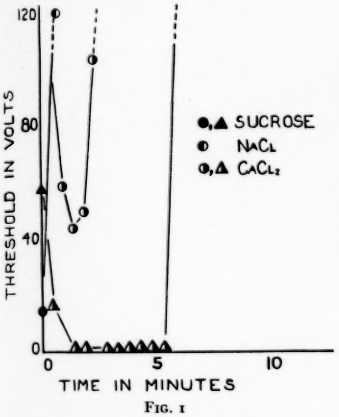

FIG. 1

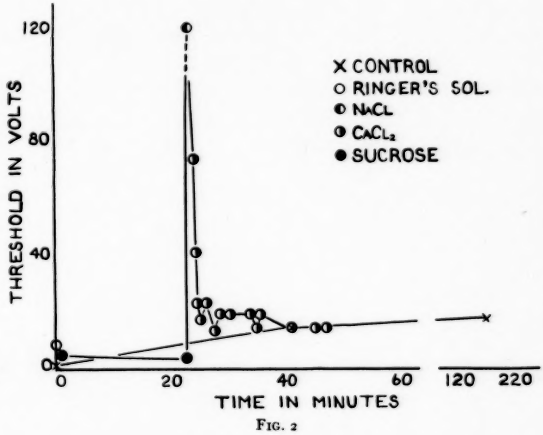

FIG. 2

FIG. 1.—Variations in threshold of a single fiber, isolated from a sucrose-treated muscle, on immersion in isosmotic $CaCl_2$ (triangles) and of a similar fiber on immersion in NaCl followed by $CaCl_2$ (circles). The points on the *y*-axis give the respective thresholds on isolation of the fibers. The broken lines indicate that the threshold was higher than 105 volts, which was the maximum voltage obtainable.

FIG. 2.—Variations in threshold in response to variations in the medium surrounding a fiber dissected in Ringer's solution, then immersed successively in isosmotic sucrose, NaCl and $CaCl_2$ (circles). Broken lines indicate that the threshold was higher than 105 volts. Crosses show variations in threshold of control fiber in Ringer's solution.

listed; but in the interests of economy, only 4 such graphs are presented (in Figs. 1 and 2). Figure 1 shows the variations in threshold of 2 fibers dissected from sucrose treated muscles and then immersed in calcium of chloride in the one case and in sodium chloride followed by a sodium chloride–calcium chloride mixture in the other. These 2 graphs represent cases indicated by asterisks in Tables 2 and 3.

Experiments similar to those outlined above were performed on muscle fibers dissected in Ringer's fluid, then transferred to isotonic sucrose solution and from there into an isotonic solution of sodium chloride (see Table 4). This is essentially a repetition, with isolated fibers, of Overton's basic experiment on whole muscles. In the whole muscles irritability is lost in the sugar solution and regained in the solution of sodium chloride. But Table 4 shows very clearly that for the isolated fibers any loss of irritability in sugar solution is never regained in the sodium chloride solution. Indeed, in 14 out of 17 cases

irritability is completely lost in the sodium chloride solution. However, in many cases the addition of a small amount of a calcium chloride solution to the fluid bathing the fibers results in a restoration of irritability; and in every instance but one, the calcium, if it produces any measurable change at all, decreases the threshold of the fiber. Figure 2 shows the variation which a single fiber undergoes on successive immersion in Ringer's solution, isosmotic sucrose, sodium chloride, and calcium chloride, as compared with a control fiber in Ringer's solution.

The experiments reported so far indicate that an isotonic solution of sodium chloride, far from restoring the irritability of muscle fibers previously immersed in sucrose solu-

TABLE 4

Threshold in Ringer's Solution (Volts)	Duration of Immersion in Sucrose (Minutes)	Threshold in Sucrose (Volts)	Duration of Immersion in NaCl (Minutes)	Threshold in NaCl (Volts)	Duration of Immersion in NaCl+ $CaCl_2$ (Minutes)	Threshold in NaCl+ $CaCl_2$ (Volts)
6	1	45	0.5	>105	2	36
1.7	3	30	1	>105	2.5	33
5	2.5	27	1	>105	2	30
2	20.5	24	0.5	>105	0.5	60
18	10	30	0.5	>105	0.5	105
15	9	30	1	39	0.5	30
9*	22	4	0.25	>105	4	14
69	1	18	2.5	90	0.5	48
0.5	88	60	1	>105	1	84
45	12.5	33	1	>105	0.5	42
12	1	7	1	12	0.5	21
6	2	54	1	>108	0.5	108
105	16	57	4	>105	0.5	105
1.5	5	6	0.5	>105	0.5	45
27	1	54	1	>105	1	105
2	27	9	0.5	>105	0.5	30
2	15	27	0.5	>105	0.5	105

* The behavior of this particular fiber is shown graphically and in more detail in Fig. 2.

tions, has the opposite effect. Following decrease or loss of irritability in sodium chloride solutions, the addition of a small amount of isotonic calcium chloride results in an increase in irritability. In these experiments, in order to avoid handling and possible injury of the fiber, the calcium chloride solution was added directly to the drop containing the fiber. One disadvantage of this procedure was that it was not possible to know the exact ratio of calcium to sodium in the resultant solution.

In a final series of experiments (Table 5) fibers were dissected in Ringer's fluid, were transferred to sucrose and then to sodium chloride solution, and finally from the sodium chloride solution to a mixture of sodium and calcium chlorides in the proportion in which they occur in Ringer's fluid. The results were thoroughly consistent. In sucrose the fibers did not lose their irritability, although in general they were less irritable than in Ringer's fluid. However, on transfer to isotonic sodium chloride solution, in all 25 instances a complete loss of irritability resulted. Then on transfer to a solution containing sodium and calcium, a return of irritability occurred.

In summary, it may be stated that all our experiments with isolated fibers show convincingly that the sodium ion has no power to restore or improve the irritability of fibers previously immersed in sugar solution. Indeed, of the three common cations, calcium alone has this power. Our results, therefore, are in direct opposition to the belief of Overton that the irritability of muscle fibers is primarily conditioned by the presence of sodium ion in the medium surrounding them.

TABLE 5

Threshold in Ringer's (Volts)	Duration of Immersion in Sucrose (Minutes)	Threshold in Sucrose (Volts)	Duration of Immersion in NaCl (Minutes)	Threshold in NaCl (Volts)	Duration of Immersion in K-free Ringer's (Minutes)	Threshold in K-free Ringer's (Volts)
1	3	21	0.5	>105	1.0	39
3.5	2.5	12	0.25	>105	1.5	66
18	13.0	30	0.25	>105	0.5	37
2	0.5	1.5	1.5	>105	1.5	75
2.5	0.5	9	0.5	>105	0.5	45
13	1.5	19	0.5	>105	0.75	34
1	1.5	28	0.5	>105	18.0	48
9	2.5	48	0.5	>105	0.75	30
1	2.0	12	0.75	>105	34.75	57
4.5	2.5	8	0.5	>105	1.5	27
3	0.5	12	0.5	>105	1.0	55.5
3.5	24.0	21	0.25	>105	0.5	9
3	2.0	15	0.5	>105	0.5	63
1	1.0	42	0.5	>105	0.25	60
10	1.0	21	0.5	>105	22.5	24
2	1.5	39	0.5	>105	16.5	30
1.5	1.0	30	0.25	>105	0.5	30
3	0.5	10.5	1.0	>105	12.0	27
45	2.0	42	0.5	>105	1.0	36
84	4.5	30	0.5	>105	0.5	30
2.5	0.5	21	0.5	>105	1.0	45
1	1.0	8	0.25	>105	3.25	27
3	2.0	42	0.5	>105	1.0	21
4.5	1.0	24	1.5	>105	1.5	30
3.0	0.25	30	0.5	>105	1.0	75

GENERAL CONCLUSIONS

Overton's experiments are reproducible, and we have no quarrel with them. However, his widely accepted conclusion that the irritability of muscle fibers is primarily dependent on the presence of sodium ion in the surrounding medium can no longer be regarded as valid. When muscles immersed in sugar solution fail to show readily observable response to electrical stimulation, this appears to be in part due to a failure of the electric current to reach individual fibers. (It may also in part be due to a failure of the conduction mechanism within the fibers.) The irritability of the isolated (cut) fiber has been shown to be dependent on the presence of calcium ion. When a fiber is isolated in sucrose solution, irritability is retained for some time, presumably because of the presence of calcium ion in the surface or cortex. Washing of such sucrose-treated fibers with solutions of sodium chloride apparently removes this calcium, for it destroys the irritability. Subsequent restoration of irritability occurs only when calcium ion is added to the medium surrounding the fibers.

SUMMARY

1. As Overton originally stated, frog muscles lose their irritability when immersed in sugar solutions and regain it on being transferred to a solution of a sodium salt.

2. The indirect excitability of the muscle depends on the presence of calcium ion rather than sodium ion. This is in accordance with the opinion of numerous older authors.

3. When muscles have been immersed for several hours in sugar solution, the individual fibers, on isolation, may be found to have retained their irritability.

4. This is taken to indicate that one factor involved in the loss of irritability in sugar solutions is the failure of the sugar solution to conduct the electric current to fibers remote from the electrodes.

5. When muscles are immersed in sugar solutions, irritability to mechanical stimulation is rapidly lost. Such irritability is restored on transfer to solutions of sodium chloride, but typically no restoration of irritability occurs in solutions of sodium citrate or oxalate. The facts with regard to mechanical stimulation are difficult to interpret. It is possible that irritability to mechanical stimulation may depend on the presence of calcium ion in the surface of the fiber, but this cannot be regarded as proved.

6. Experiments with isolated fibers show clearly that, following immersion in sugar solutions, subsequent treatment with solutions of sodium chloride causes only a loss of irritability.

7. Calcium ion increases the irritability of isolated muscle fibers previously immersed in sugar solution, and it restores the irritability of isolated fibers which have become non-irritable following treatment first with sugar and then with sodium chloride solution.

8. The irritability of isolated fibers, and presumably of muscle fibers *in situ*, seems to be dependent on the presence of calcium ion rather than sodium ion.

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THE EFFECTS OF PERIPHERAL INHIBITION ON THE MUSCLE ACTION POTENTIALS OF THE CRAB

(Five figures)

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IT HAS been shown previously (van Harreveld and Wiersma, 1937) that the muscular contractions of decapod crustacean muscles can be inhibited peripherally by the stimulation of certain nerve fibers. In most muscles only one inhibitory fiber has been found, and this fiber can inhibit the contractions caused by any of the from one to four motor fibers which supply the muscle. Recently it has been shown that in the crab, *Cancer anthonyi*, the opener muscle[1] is innervated by two inhibitory fibers both of which inhibit the contractions caused by the stimulation of the single motor axon supplying it (Wiersma, 1941). This situation has opened the possibility of investigating in one muscle the effect of inhibition by two different inhibitory fibers on the muscle action potentials. In the opener of the crayfish, *Cambarus*, where only one inhibitor is present, it has been found that the reduction of the action potentials is by no means necessary for inhibition of the mechanical contraction (Marmont and Wiersma, 1938). Only when the inhibitory impulses arrive at the muscle just before the excitatory ones is a pronounced reduction of the action potentials obtained. This reduction of the action potentials has been termed "supplementary inhibition." No reduction is visible when the inhibitory impulses arrive slightly later (simple inhibition).

One of the two inhibitors innervating the opener in the crab also inhibits the closer, which has a double motor innervation. As a result, the effect of inhibition could be investigated on the two types of action potentials exhibited by a doubly innervated muscle.

METHOD

The technique used to prepare single motor and inhibitory nerve fibers has been described before (van Harreveld and Wiersma, 1936, 1937) and does not need to be repeated here. Only the walking legs of the crabs were used in the present investigation, as claws of *Cancer* are too clumsy to handle, and, furthermore, fatigue occurs much sooner in them. The nerve bundle in the meropodite mainly consists of sensory fiber bundles. The efferent fibers usually are found in two small bundles, one of which contains the two motor axons of the closer muscle, while the other, which is usually Y-shaped, contains the remaining six efferent fibers. Separating the two branches of this Y usually isolates the opener-stretcher motor axon and the opener and the stretcher inhibitors from the two bender motor axons and the "common" inhibitor. This "common" inhibitor has been shown to innervate at least five muscles in the leg, among which are the four distal muscles (Wiersma, 1941). When the inhibitory effect of this fiber on the closer or opener is

[1] The names "opener" and "closer" will be used in the present paper for the abductor and the adductor muscles, moving the dactylopodite of the chelipeds and the walking legs. The innervation is the same in these legs.

investigated, it is not obligatory to separate it from the two bender axons, since the action potentials of the bender have been found to be undetectable when leading off from the closer or the opener muscles. For the same reason the action potentials of the stretcher do not disturb the opener action potentials. The stretcher inhibitor usually was left attached either to the opener-stretcher motor axon or to the "true" opener inhibitor. During the experiments with the two inhibitors of the opener the "true" and the "common" inhibitor were always differentiated from each other by testing them against the closer contraction.

Two methods have been used to obtain differences in the time of arrival of the excitatory and inhibitory impulses at the muscle. The first one has been described before (Marmont and Wiersma, 1938). In this method an induction coil with one primary and two secondaries is used. In this way both fibers are stimulated at the same time, the variable factor used to obtain differences in the arrival time being the distance the impulses have to travel through the nerve fibers. When this method is employed, the stimulating electrodes have to be moved for each difference recorded, and this frequently results in mechanical injury which restricts the area receptive to stimulation. The second method developed to circumvent these difficulties enables a series of two stimuli to be given with a variable interval of time between them. In this method the electrodes were placed at the same level, and moving them was unnecessary. Frequencies ranging between 20 and 100 per second were used. The maximal variation between the two shocks that could be obtained with this apparatus was 8.3 milliseconds; thus, at a basal frequency of 60 per second it was possible to cover the complete cycle. A more detailed description of the apparatus is given in the Appendix, by G. Keighley, who built the stimulator.

The usual procedure employed in the experiments was to stimulate for 6 seconds. During the first 2 seconds of a 6 seconds' stimulation the motor fiber alone was stimulated; during the next 2 seconds the inhibitor was also stimulated (either before or after each excitatory stimulus); and during the final 2 seconds again only the motor axon was stimulated.

The action potentials were recorded by means of a Mathews oscillograph and amplifier. The leading-off electrodes were silver–silver chloride wires which were covered with cotton wool. In some instances records of the mechanograms were simultaneously obtained by attaching a strong-springed auxotonic lever (with a mirror attached) to the dactylopodite.

THE EFFECTS OF THE OPENER INHIBITOR ON THE ACTION POTENTIALS OF THE OPENER MUSCLE

Although no differences in the mechanical inhibition can be detected whether in a series of stimuli the individual impulses in the true opener inhibitor arrive at the muscle immediately before or immediately after the excitatory ones, the difference of the effects on the action potentials is very pronounced. As in the crayfish, inhibitory impulses which are started immediately before the excitatory ones invariably and often markedly reduce the action potentials, and this effect is not perceived when the inhibitory impulses are started after the excitatory ones. The maximal amount of reduction possible is usually obtained when the inhibitory impulses precede the motor impulses by one millisecond (Fig. 1 *A*, *B*, and *C*), using the double-pulse stimulator, or with a distance of 4 mm. between the electrodes, the inhibitory ones in front, using the two secondary coils.

With either method considerable variation was observed between different preparations (cf. Fig. 1, *D*). The range of time-intervals which will cause a noticeable reduction of the action potentials varies also in different preparations. Sometimes the range was limited to about 7 milliseconds (Fig. 1, *A*), while in other preparations it extended over a much greater range (Fig. 1, *B*, *C*, and *D*). The maximum amount of reduction obtainable varied in different preparations, being at most 75 per cent (Fig. 1, *A* and *B*) and at least 50 per cent (Fig. 1, *C* and D). There was no obvious relation between the maximum amount of reduction and the range of time over which this supplemented inhibition could be produced. In the crab, as in the crayfish, the reduction of the action potentials at the onset of a supplementary inhibition is rapid, and in most cases the action potentials soon attain a level which remains fairly constant for the rest of the inhibitory period.

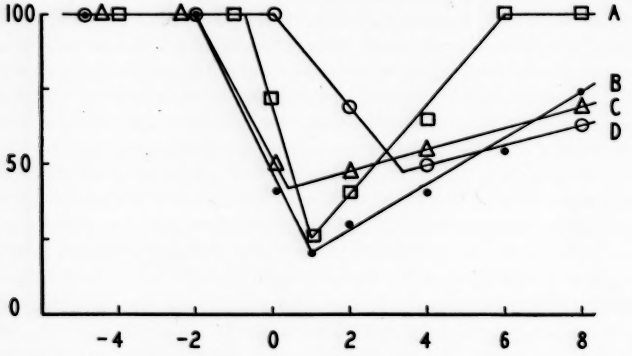

FIG. 1.—Diagram showing the effect of the relative time of arrival of the inhibiting and exciting impulses upon the height of the action potentials in four different preparations. Stimulation frequency of the true opener inhibitor and the opener excitator was 30 per second. The abscissa represents the times in milliseconds that the inhibiting impulses were started in advance of the exciting ones; the ordinate gives the height of the reduced action potentials in percentage of the normal, unreduced height.

On the release from inhibition in the crayfish the reducing effect of the supplementary inhibition disappears immediately after the last inhibitory impulse has produced its effect; but in most of the crab preparations the initial action potentials after release are still considerably depressed, compared with the unreduced height, although even the first uninhibited one always shows a considerable growth (Figs. 2, *B*, and 3, *B*). The return to normal is usually more or less gradual (Fig. 2, *B*). There is thus established during the time of simultaneous excitation and inhibition a state of depression of the height of the action potentials which outlasts the inhibitory stimulation. Although this effect presents itself most clearly after supplementary inhibition, it is also present during simple inhibition. Here it shows itself by a more or less pronounced prevention of the growth of the action potentials during inhibition. In some preparations the growth is totally suppressed during simple inhibition, and in these the stimuli following the release from inhibition cause a pronounced increase in the size of the action potentials (Fig. 2, *A*); in other cases the depression can scarcely be found at all (Fig. 3, *A*). There is an absolute agreement in any one preparation between the effects produced after both kinds of inhibition. If the growth after supplementary inhibition is pronounced, it will also be

pronounced after simple inhibition; and if it is small in the first case, it will be too small to be seen in the second. This may be seen in Figures 2 and 3, respectively.

In preparations in which this effect on the growth of the action potentials is very pronounced, the action potentials during simple inhibition are easily distinguished from those which are not inhibited, and the decline of the supplementary inhibited action potentials may go on for a considerable time after the initial rapid reduction. That this effect is present to a certain extent in every case may be shown by the impossibility of obtaining rebound action potentials in the crab. In the crayfish long periods of supplementary inhibition are followed, on release, by action potentials of unusual height. This is explained by continued facilitation and at the same time prevention of fatigue of the action-potential mechanism. In the crab such a rebound was never obtainable, the action potentials never being larger than those before the inhibitory period.

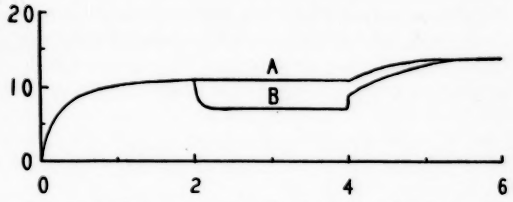

FIG. 2.—Diagram showing simple and supplementary inhibition in a preparation in which the action potential growth is greatly influenced by inhibition. *A* = simple inhibition: during inhibition complete stoppage of growth. *B* = supplementary inhibition: pronounced growth of the action potentials after release of inhibition. Ordinate = height of action potential in millimeters. Abscissa = time in seconds, inhibition from 2 until 4 seconds after start. Curves obtained by drawing a line joining the tops of the action potentials.

After this effect of inhibition on the growth of the action potentials during simultaneous excitation and inhibition had been found in the crab, it was considered likely that inhibitory stimulation by itself would have a very pronounced effect on the growth of the action potentials in a subsequent excitation. Such an after-effect of inhibition has been found to exist to a marked degree in the crayfish. In that animal prolonged stimulation of the inhibitory fiber alone (1 minute or more) is followed by a state in which subsequent stimulation of the motor fiber results in muscle action potentials the growth of which is slower than normal. A series of experiments was completed in the crab in which the inhibitor alone was stimulated for various durations, ranging from a few seconds to several minutes. After the completion of this inhibition, the excitator was immediately stimulated. Remarkably, in none of the experiments was there any pronounced effect on the growth of the action potentials, the differences found always being within the limits of experimental error. Thus there does not seem to be a direct relation between the after-effect as described in the crayfish and the previously mentioned depression of the growth of the action potentials in the crab. It may be mentioned that in both the crab and the

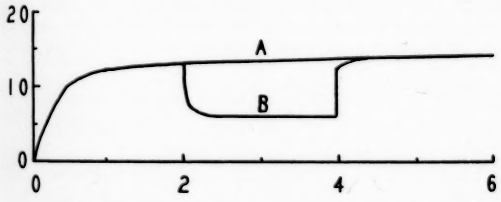

FIG. 3.—Same as Fig. 2, but preparation shows scarcely any influence of inhibition on the action-potential growth. *A* = simple inhibition: no visible effect. *B* = supplementary inhibition: a slight depression on release, which disappears quickly.

crayfish the effect of a previous period of inhibition makes itself observable by a rather marked depression of the mechanical contraction, which continues for some time after the inhibitory stimulation has been applied.

Since it was possible with the apparatus to give the two stimuli at different frequencies, the influence of different frequencies on the amount of supplementary inhibition could also be investigated. With low frequencies the facilitation of the action currents is less, and thus it is not surprising that the absolute height of the action potentials during supplementary inhibition is smaller at low than at high frequencies of stimulation. However, when the reduction of the action potential expressed as a fraction of the height of the unreduced action potential (this latter factor becomes more uncertain the more the reduction is due to depression of the facilitation) is plotted against the time-interval of

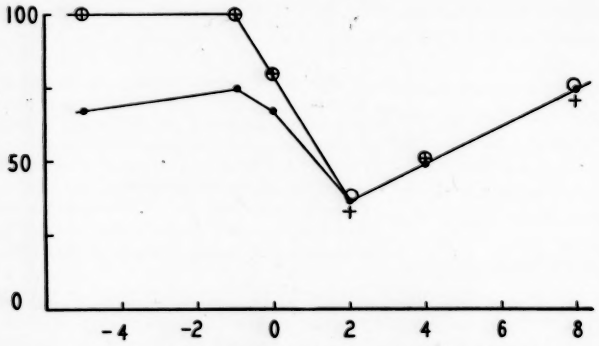

FIG. 4.—Diagram showing the effect on reduction of the action potential of the time of arrival of the inhibitory and excitatory stimuli in one preparation, at different frequencies. Co-ordinates, same as in Fig. 1. ○ represents the values obtained at a frequency of 30 per second; + represents the same at stimulation frequency of both fibers at 50; and ● represents the same at 100 per second.

the two shocks, the figures show a remarkable constancy in all cases below a frequency of 60 per second (Fig. 4). In the frequencies around 60 "simple" inhibition gradually changes into "supplementary" inhibition, and with still higher frequencies only supplementary inhibition can be produced. There will be, of course, as long as the distance between shocks is not too short, a minimum of reduction at values where the inhibitory impulse is set up just after the excitatory one. The fact that changes in frequency have no influence on supplementary inhibition shows that the facilitation of the inhibitory effect must develop at similar rates to that of the excitatory one. Similar results have been obtained in the opener of *Cambarus* using different frequencies of supplementary inhibition.

THE EFFECT OF THE COMMON INHIBITOR ON THE ACTION POTENTIALS OF THE OPENER AND THE CLOSER

The effect of the common inhibitor on the opener action potentials is markedly different from that of the true opener inhibitor. Under no circumstances is there a real supplementary inhibition, for, even in preparations in which the true opener inhibitor causes very marked reductions of the action potentials when the impulses are correctly

timed, the effect of this same time-interval or, for that matter, any other time-interval between the impulses of the excitator and the common inhibitor fails to produce any significant reduction. Figure 5, *A* and *B*, shows a comparison of the effects of the common and the true inhibitor in the same preparation.

However, as can be seen in Figure 5, *A*, stimulation of the common inhibitor during excitation is not without some effect on the action potentials. Depending on the preparation, a more or less pronounced stoppage of the growth of the action potentials takes place during this inhibition. It can be shown, however, that this reduction is correlated with the reduction during simple inhibition by the true inhibitor. Preparations in which simple inhibition by the true inhibitor fiber causes a stoppage of growth also present this type of picture when the common inhibitor is stimulated. Since the differences obtained are small, it is impossible to state whether the amount of suppression of growth is the same for stimulation of the two inhibitors. If a difference is present, however, it is very slight. The growth of the action potentials after release from inhibition by the common

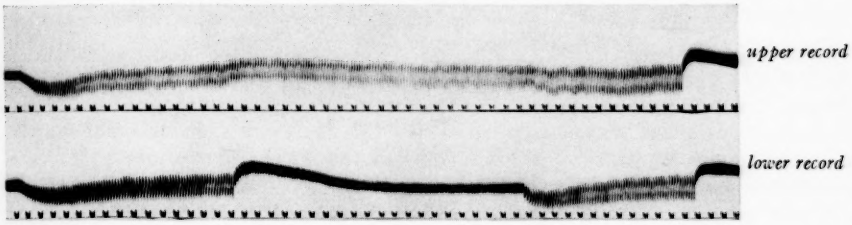

FIG. 5.—Action-potential records of one preparation of the opener of *Cancer* showing the difference of the influence of the common and the true opener inhibitor, when the inhibitory impulses were started 1 millisecond before the excitatory ones. Upper record shows stimulation of the common inhibitor: only very insignificant reduction. Lower tracing gives stimulation of the true opener inhibitor: maximal supplementary inhibition. Time in 1/10 second. Stimulation frequency, 50 per second.

inhibitor is dependent on the amount of reduction of the facilitation during inhibition, just as is the growth of the action potentials after release from simple inhibition by the opener inhibitor.

The common inhibitor fiber is only slightly less efficient than the true opener inhibitor in producing mechanical inhibitions. In both cases the contraction is usually completely suppressed by the inhibitory stimulation, and after release from inhibition the contraction is faster than the control contraction. However, in comparison with *Cambarus* this facilitation is not very pronounced. Certain factors, as, for example, fatigue and the length of time during which an opener contraction is facilitated after a foregoing contraction, play an important role in this respect. This makes the experiments less exact than in *Cambarus*, where these factors are of much less influence. It may suffice to state in this regard that, on release, the contraction in *Cancer* may be either faster, equal, or slower than a control contraction given at the same time after the initial contraction as the release contraction. It is likely that slow contraction is correlated with slow growth of the action potentials on release, and vice versa.

The effect of the common inhibitor on the action potentials of the closer muscle resembles that of the common inhibitor on those of the opener but is more pronounced in

some preparations. Whereas no real reduction of the opener action potentials has been obtained by the stimulation of this fiber, in a few preparations the closer action potentials may show a noticeable reduction during inhibition. In some cases as much as a 30 per cent reduction has been noticed both in the slow and in the fast action potentials. As a rule, the results of each preparation are uniform. If a reduction is obtained in a preparation, it is observed at all time-intervals and is of equal magnitude throughout. The frequency of stimulation also has no marked influence, although with high frequencies (80–100) the reduction may be quicker and somewhat more pronounced than with low frequencies (20–40). In most preparations, however, the depression of the action-potential growth is only sufficient to slow down or stop the growth, and in some even this effect is hardly visible. In the cases where reduction is present, there is always a pronounced subsequent growth of the action potentials on release, as should be expected from the foregoing results. Thus it appears that the common inhibitor affects the action potentials of both the closer and the opener muscles in the same manner but is more effective on the former.

DISCUSSION

The difference in the reduction of the action potentials of the opener muscle by the two inhibitors is remarkably clear in the curves obtained. It might be argued that the absence of supplementary inhibition by the common inhibitor is due not to a difference in the mechanism but to a special peculiarity of this fiber, which conducts inhibitory impulses to the places where they act in such a way that these different places are reached at various times in relation to those at which the motor impulses arrive. A number of arguments may be raised against this. First, there is no anatomical evidence in the arrangement and divisions of the fibers for any such peculiarity of the common inhibitor. Second, even if such a peculiarity existed, it would not explain the complete absence of any influence of the interval between exciting and inhibiting stimuli on the amount of reduction of the action potentials. Third, pronounced reduction should always be obtained with high frequencies, since the chances of supplementary inhibition at any given point of the muscle then would be greatly increased. However, as high frequencies of stimulation were without much influence on the reduction of the action potentials by the common inhibitor, it must be considered that the common inhibitor is without the ability to give supplementary inhibition.

Another possibility would be that fatigue of supplementary inhibition was very quick on stimulation of the common inhibitor. If this were the case, however, at least a temporary supplementary inhibition would be expected in a fresh preparation.

It is thus concluded that the process of supplementary inhibition and the process responsible for the depression of growth of the action potential, which is not dependent on the time relations, are different. In the crayfish, in which only the first can be found, it has been concluded that inhibition has no influence on the process of action-potential facilitation but that supplementary inhibition results when a block is established in the transmission process between nerve impulse and the process underlying the action potential. This block prevents the fatigue of the action-potential mechanism; and as soon as it is removed, high rebound potentials occur which show that the facilitation process has continued. The absence of such rebound potentials and the fact that in some preparations all intervals of arrival times lead to a stoppage or reduction of the growth point to the conclusion that in the crab inhibition has a depressing effect on the process of facilita-

tion. The magnitude of this effect is, however, different in different preparations and often is very small. Consequently, it is wrong to conclude that in the crab the action of inhibition on the facilitation of the action potentials represents the main factor of inhibition. In the crab, as in the crayfish, the block between the action potentials and the contraction is the main factor in the mechanical inhibition, and in supplementary inhibition it is the block in the transmission process between the nerve impulse and the muscle action potential which always plays the main role. In the majority of cases in the crab it is even true that facilitation of the action potentials goes on notwithstanding inhibition, although the total amount of this facilitation is always at least somewhat diminished during inhibition. As already has been said above, this reduction in facilitation is only of minor significance in the mechanism of inhibition.

It is of interest to note that the opener inhibitor, which must be one of the inhibitors which acts constantly during certain reflexes, produces supplementary inhibition, whereas the common inhibitor, which can play only a very minor part during normal movements, lacks this faculty. It is not likely, however, that there is a relation between the effectiveness of inhibition, on one hand, and the possibility of supplementary inhibition, on the other. Mechanically the inhibition of the opener in the crab by the true opener inhibitor and the slow closer contraction by the common inhibitor have about the same efficiency, whereas supplementary inhibition is found only in the former muscle.

SUMMARY

The influence of stimulation of prepared single inhibitory axons on the muscle action potentials during contraction has been studied in two muscles of the leg of the crab, *Cancer anthonyi*.

In the opener, which receives one exciter and two inhibitors, it was found that the two inhibitors differ in their effects. Stimulation of one of them—the "common" inhibitor—has under no circumstances a pronounced effect on the muscle action potentials. Stimulation of the other—the "true" opener inhibitor—can lead to a considerable (up to 75 per cent) reduction of the potentials if the inhibitory impulses are made to arrive at the muscle just before the excitatory ones. This reduction is similar to, but differs in some respects from, that obtained in the opener muscle of the crayfish.

In the closer, which is innervated by two motor axons and by the "common" inhibitor, the effect of inhibition was studied for both contraction types, which have different action potentials. No reduction of the potentials dependent on the arrival time could be obtained. It is concluded that the "common" inhibitor does not give supplementary inhibition.

A small depression of the growth of the action potentials during any inhibition was found in the crab in contrast with earlier results in the crayfish. It is pointed out that this reduction can have only a minor part in inhibition and that the inhibition of the contraction does not depend in any case on reduction of the action potentials.

A method to obtain two shocks with a variable interval at different frequencies of stimulation is described.

APPENDIX

THE DOUBLE-PULSE STIMULATOR

GEOFFREY KEIGHLEY

The two stimulating pulses with a variable interval between them were obtained by rectifying the output of a stimulator which gave rectangular waves, the frequency and duration of which could be varied independently. The output of this rectangular wave generator was coupled through an audio-transformer with a center tapped secondary to a full-wave rectifier consisting of two triodes. The two outer taps of the secondary were tied directly to the plates; the center tap was divided into a circuit of two legs; and each of these led through a potentiometer to the cathodes. The grids of the two tubes were tied to the plates, the filaments heated by a storage battery. At the beginning of each rectangular wave applied to the rectifier there is a pulse in one direction which passes through one rectifier tube; at the end of each rectangular wave there is a pulse in the other direction which passes through the other rectifier tube. The output is taken from the common terminal of the two potentiometers (the center tap of the transformer) and the two movable taps; the pulses appearing between this common tap and the two variable ones are of the same polarity. The interval of time between the two pulses depends on the duration of the rectangular wave applied to the rectifier, as they are the resultants of the on-off portions of this wave; the interval between each pair of pulses is set by the frequency of the applied rectangular waves. The two potentiometers control the strength of each pulse separately.

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MODIFICATION OF DEVELOPMENT IN THE SAND DOLLAR BY NaCNS AND Ca-FREE SEA WATER[1]

(Thirty-four figures)

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Hopkins Marine Station and Wayne University

IN RECENT years the dynamics of echinoderm development has attracted the attention of such noted investigators as Runnström (1914, 1915, 1917, 1918, 1933, 1935), Hörstadius (1935, 1937, 1939), Lindahl (1933, 1936), von Ubisch (1925, 1929), Child (1915, 1916*a*, 1916*b*, 1929, 1936*a*, 1936*b*, 1940), and many others. The Runnström school at Stockholm has been inclined to look upon the pattern in the early development of the echinoderm egg as the result of an interaction of two opposed material gradients. According to these workers, the animal gradient, extending from the animal to vegetal pole of the egg, tends to ectodermize the larva, while the opposing gradient, from the vegetal to animal pole, tends to entodermize the larva.

Child, on the other hand, has shown with the aid of vital dyes a reduction gradient from the animal to vegetal poles in the early stages. This, together with numerous experiments on larval modifications, has led to the belief that a polar activity gradient may be the primary developmental pattern. He suggests (1936*a*) that the different fields (such as the entoderm field of the late blastula) arise through a process of partial physiological isolation from the primary, or polar gradient.

The work here undertaken deals with the modification of development with NaCNS and Ca-free sea water as test agents in an attempt to throw additional light on the subject of early developmental patterns.

MATERIAL AND METHODS

Only the eggs of the sand dollar, *Dendraster excentricus*, were used in these experiments. Since the work was carried out during the height of the breeding season (June–August), 95–100 per cent fertilization was the rule when a few drops of sperm suspension were added to eggs freshly taken from ripe females. The cultures consisted of approximately 200 eggs in 150 cc. of solution. They were kept in covered finger bowls on a water table. With all tests were run parallel control lots in normal sea water. The eggs were always washed after fertilization, and when removed from the test solutions, by pipetting them through several changes of sea water. During the course of the experiments the temperature varied from 13° to 18° C.

In making up the solutions of NaCNS, Lindahl's method (1933) was followed. This consisted of adding various quantities (5–50 cc.) of the agent in isotonic strength (0.54 M) to 100 cc. of Ca-free or normal sea water. Ca-free sea water was made up, with slight modifications, according to Herbst's formula.[2]

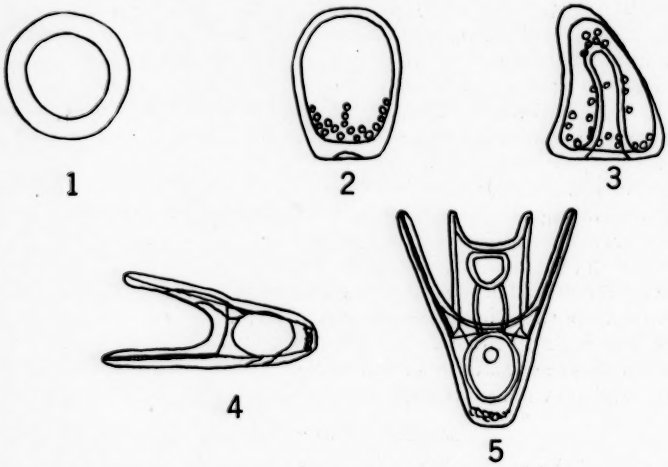
[1] The writer wishes to express his appreciation to Dr. W. K. Fisher, director of the Hopkins Marine Station, Pacific Grove, California, for the laboratory space and facilities which made this work possible. To Dr. C. M. Child the writer is especially indebted for many valuable suggestions and criticisms during the entire course of the work.

[2] Distilled water, 10,571 cc.; NaCl, 307 gm.; KCl, 8 gm.; $MgSO_4$, 66 gm.; $NaHCO_3$, 2 gm.

NORMAL DEVELOPMENT

In most respects the early development of the sand dollar parallels that of the sea urchin, but there are some differences (Child, 1940). For comparative purposes certain of the normal stages are described briefly in the following paragraphs.

The fertilization membrane normally appears within a few minutes after fertilization, and cleavage takes place within this membrane. Cleavage is total and very similar to that of the sea urchin. By 7–8 hours after fertilization the middle blastula stage is reached (Fig. 1). Here the cells are quite small, the epithelium is smooth, and the wall of the blastula is still fairly thick. Cilia are beginning to appear at this stage, and the embryo may be revolving within the fertilization membrane. By 12 hours after fertiliza-



FIGS. 1–5.—Normal development of the sand-dollar larva from 7 to 60 hours after fertilization at a temperature of approximately 15° C.

tion (Fig. 2) most of the embryos are top-swimming, mesenchyme has appeared, and the first signs of gastrulation are noticeable. Except for the extreme vegetal region the walls of the blastocoel have thinned considerably.

At 17 hours after fertilization bilaterality begins to be evident with a flattened ventral and a rounded dorsal surface. At 24 hours (Fig. 3) bilaterality is distinct, the long archenteron is being directed toward the ventral surface, the oral lobe is directed ventrally, and from a ventral view the first indications of the coelomic pouches may be seen. At this stage the earliest indications of the anal arms, which arise laterally in the posterior or anal regions of the ventral surface, may also be found.

By 45–48 hours after fertilization the larva has become a fairly well-developed pluteus (Fig. 4, lateral aspect). The anal arms are quite long, while the oral lobe has developed arms and become narrower. The skeleton, which was first noted as minute skeletal rods at about 28 hours, is fairly well developed at this stage. A stomodaeum had appeared at approximately 30 hours. At 60 hours (Fig. 5, anal view) the anal and oral arms are

longer, and the oral lobe is narrower than in the 45–48-hour larva. The mouth is large and, together with the gut, is usually undergoing rhythmic contractions. At this stage, if no feeding is undertaken, the animals begin to die.

EXPERIMENTAL

1. *The effect of NaCNS on developmental pattern with treatment before fertilization.*— Twenty-four experiments were performed in which unfertilized eggs were submitted to concentrations ranging from 10 to 50 cc. of isotonic NaCNS per 100 cc. of Ca-free sea water for 12–24 hours before fertilization. The eggs were then fertilized and allowed to develop in normal sea water. Parallel experiments were run with normal and Ca-free sea water alone.

The most interesting results were obtained when the unfertilized eggs were exposed for 12 hours to 20 cc. of isotonic NaCNS plus 100 cc. of Ca-free sea water. Through this treatment the rate of cleavage was retarded when the eggs were returned to normal sea water and fertilized. Observations of such cultures 24 hours after fertilization showed that 30–40 per cent of the living embryos had failed to gastrulate or give off mesenchyme into the blastocoel (Figs. 6 and 7). Most of these mesenchyme-free forms showed considerable movement, as well as some degree of ventrodorsal flattening (Fig. 7). By 60 hours after fertilization most of these forms were still without mesenchyme or gut (Figs. 8–12) and appeared as transparent, pigmentless forms without skeleton but in many cases taking on the general shape of a pluteus (Fig. 12). These larvae may be said to be completely "ectodermized." They usually developed a stomodaeum which opened into the cavity of the blastocoel. In some cases the stomodaeum was considerably enlarged and occupied a central position on the ventral surface.

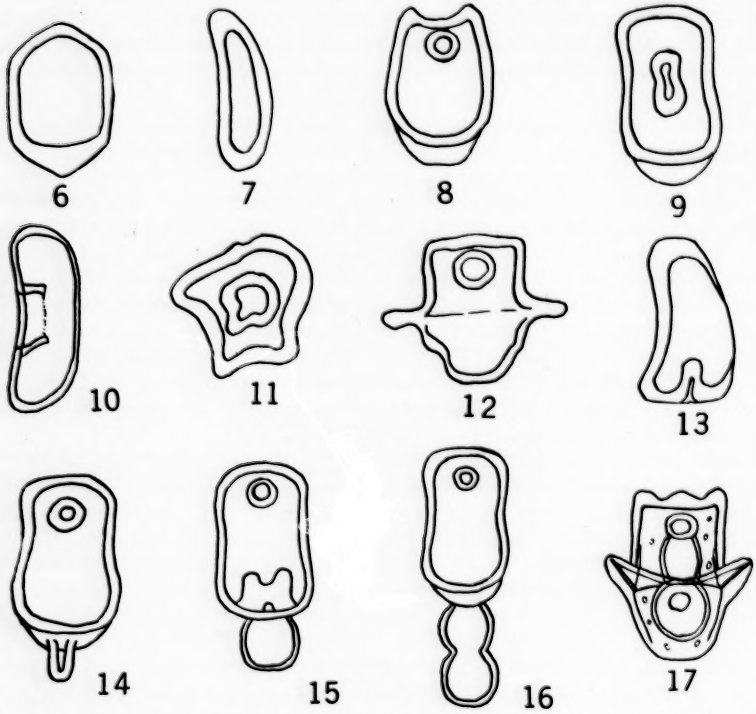
A number of the forms appearing in the test cultures were only partly ectodermized. Some of these developed a very small evaginated or invaginated gut but no mesenchyme (Figs. 13 and 14), while others developed a diminutive gut with a bit of mesenchyme. In a number the gut appeared late in development as an evagination from the presumptive anal region of a mesenchyme-free larva (Figs. 15 and 16). In some there was a partial invagination of such an evaginated gut (Fig. 15).

Many forms which invaginated but had less mesenchyme than normal developed in these cultures. These developed into plutei showing less differentiation of the oral lobe, shorter anal arms, less skeleton, and less pigment than the normal pluteus (Fig. 17). But not all of the eggs submitted to the test conditions resulted in ectodermized larvae. Some developed into normal plutei, while others showed *excessive* mesenchyme and gave other indications of inhibition, such as short anal arms and failure of the oral lobe to differentiate. There was an occasional exogastrula, and some of the eggs developed no farther than mesenchyme-filled blastulae.

In general, it was found that as the concentration of the test solution and the exposure time were increased the percentage of ectodermized forms decreased and more inhibited forms with surplus mesenchyme appeared. Concentrations of 40 cc. of isotonic NaCNS per 100 cc. of Ca-free sea water for 18 hours and 50 cc. of isotonic NaCNS per 100 cc. of Ca-free sea water for 12 hours preceding fertilization gave no mesenchyme-free forms but chiefly inhibited larvae with excess mesenchyme. In these concentrations many of the eggs failed to cleave or showed aberrant cleavages.

Eleven experiments were performed in which unfertilized eggs were submitted to

various concentrations of NaCNS in normal sea water. If the concentration was high enough and the exposure time sufficient, the eggs showed a retardation of cleavage rate and the development of inhibited forms with surplus mesenchyme when fertilized and returned to normal sea water. No ectodermized forms were produced, while many eggs failed to cleave at all or cleaved aberrantly with this treatment. Eggs exposed to Ca-free sea water alone before fertilization and then returned to normal sea water for fertilization



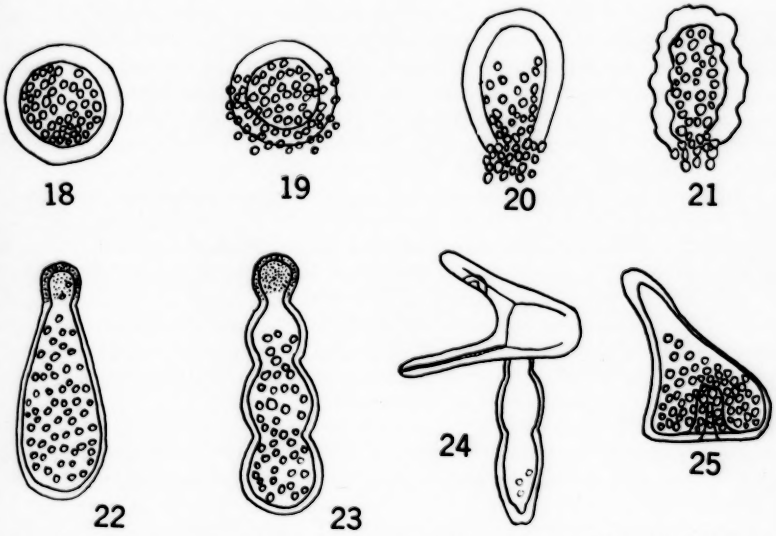
FIGS. 6–17.—Ectodermized forms of the sand-dollar larva resulting from treatment of the unfertilized egg with NaCNS in Ca-free sea water.

and development behaved little differently from the controls. Many experiments showed that eggs from different females behaved somewhat differently in that certain lots could be fertilized following longer periods after removal from the ovary than others. In general, it was found that eggs showed approximately 60 per cent cleavage if fertilized 24 hours after being removed from the ovary.

2. *The effect of exposure to NaCNS in normal sea water after fertilization.*—Twenty-six experiments were carried out in which fertilized eggs (1–2 cells) were submitted to solutions of NaCNS in normal sea water. In most cases the eggs were washed and returned to normal sea water after a few hours' exposure to the test agent.

When the concentrations were low (5 cc. of 0.54 M NaCNS plus 100 cc. of sea water) and exposure continuous, blastulae appeared which were entirely, or almost entirely, filled with cells (Fig. 18). Approximately 80 per cent of these invaginated, although the process was considerably retarded. When left in this solution, development seldom went past an inhibited gastrula stage. The remaining approximate 20 per cent developed into entexogastrulae. Return to sea water after 50 hours in the test solution resulted in slight recovery of the oral lobe, gut, and ventral side.

When the concentrations were high (40 cc. of 0.54 M NaCNS plus 100 cc. of sea water), the effect of the agent was much more noticeable. Development was much slower, and many aberrancies in the cleavage pattern were noted. Cytolysis was common.



FIGS. 18–25.—Entodermized and recovery forms resulting from treatment with NaCNS in normal sea water after fertilization.

After 12 hours in this solution the developing forms appeared as thick-walled blastulae with cells escaping internally and externally from almost all surfaces (Fig. 19). If the eggs were left in this solution, they usually cytolyzed within 24 hours. In all probability they had become anaxiate, and ventrodorsality had been obliterated long before this time.

When eggs were treated with less toxic solutions (20–30 cc. of 0.54 M NaCNS plus 100 cc. of sea water) for 24 hours, slow-moving thick-walled bottom forms with cells escaping from the vegetal pole resulted in 65–95 per cent of the cases (Fig. 20). (The remaining forms usually appeared as mesenchyme-filled blastulae.) If left in this concentration, they became highly irregular in form (Fig. 21) and soon cytolyzed. If returned to normal sea water after the 24-hour exposure period, they developed, in the vast majority of cases, into typical exogastrulae, which were usually filled with cells and showed

the ectoderm reduced to a radially symmetrical knob (Figs. 22 and 23). Such extreme exogastrulae may be said to be almost entirely "entodermized."

The treatment with solutions of NaCNS during early development often resulted in considerable recovery of the ectoderm and ectodermal structures on return to normal sea water if the concentrations were not too severe and the exposure period too long. Exposure for the first 24 hours to 10 cc. of NaCNS plus 100 cc. of sea water gave approximately 45 per cent exogastrulae when the eggs were returned to normal sea water. These exogastrulae were different from those produced with the higher concentrations in that the ectoderm was considerably larger and showed considerable differentiation (Fig. 24). Among these were forms with partial entodermal invagination of either the tip of the evaginated gut, indicating differential recovery, or of the basal gut region, indicating the lower susceptibility of that part of the entoderm. With this treatment many forms gastrulated but showed an increased amount of mesenchyme. In a few some degree of differential recovery was evidenced by the early development of an elongated apical region (Fig. 25). In these experiments, as well as in others in which the treatment was not too severe, a number of normal, or almost normal, plutei developed. These were usually top-swimming, while the affected forms were usually confined to the bottom of the culture dishes.

In most of the experiments there were varying numbers of permanent blastulae which were filled with cells or cellular debris and were apparently completely anaxiate, as well as other grossly inhibited forms, depending in a large part on the severity of the treatment. When the initial exposure was 6–8 hours in 40 cc. of NaCNS plus cc. of sea water, only a very few, if any, normal plutei developed. Most of the resulting forms showed an inhibited oral lobe and excess mesenchyme. One or both anal arms were often inhibited; and cell-filled blastulae, as well as exogastrulae, were to be found. Anal arms, when present, were commonly asymmetrical, askew, or at aberrant angles. Aberrant larvae with surplus skeleton were not uncommon.

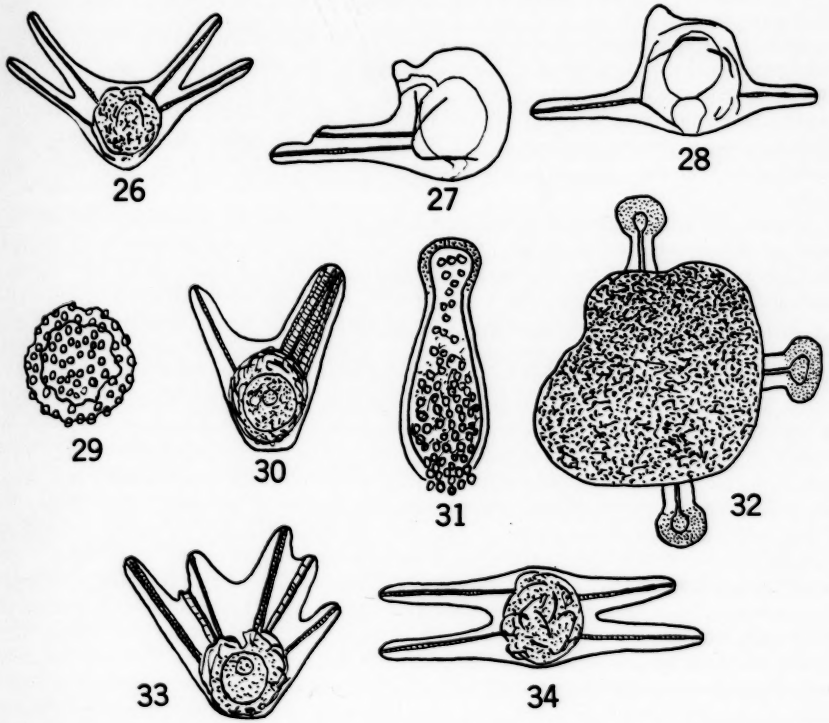
3. *The effect of exposure to NaCNS in Ca-free sea water after fertilization.*—When fertilized eggs were allowed to develop in solutions of NaCNS in Ca-free sea water for short periods and then returned to normal sea water, the resulting pattern of development was quite different than when the test solution was NaCNS in normal sea water.

An initial exposure of 4 hours to 20 cc. of 0.54 M NaCNS plus 100 cc. of Ca-free sea water showed early blastulae slightly retarded, as compared with controls. Removal to normal sea water at this time resulted, after 45 hours, in plutei which showed marked aberrancies. In general, the oral lobes had developed very little or not at all, while almost all larvae showed an excess amount of skeleton. Many possessed additional anal arms at various angles (Fig. 26), although in some only one large anal arm appeared. When two anal arms developed, they were often fused (Fig. 27), asymmetrical, askew, parallel, or at angles ranging up to 180° (Fig. 28). Few, if any, exogastrulae appeared with this treatment.

An initial exposure of 8 hours to the above test solution produced even more drastic effects. Removal from the test solution at this time showed forms which resembled early middle blastulae but which were irregular in appearance, often ruptured, and were shedding cells from all surfaces (Fig. 29). Many of these forms died; but those that lived developed into larvae showing an excess of mesenchyme, strongly inhibited oral lobes, and often an enormous surplus of skeleton (Fig. 30). Anal arms, when present, were usually asymmetrical, askew, duplicated, or at aberrant angles. Many exogastrulae with

a distorted ectoderm appeared with this treatment, while a number of grossly inhibited forms with only stubs of anal arms or no anal arms at all were likewise to be found.

Ten and one-half hours' exposure to this same test solution gave wrinkled, ruptured, middle blastulae which showed no movement and a loss of cells from all surfaces. Many were dead or dying. Fourteen hours after return to normal sea water those that survived the treatment appeared as thick-walled forms losing cells from the vegetal region. These



FIGS. 26–34.—Various aberrant forms with distorted patterns resulting from treatment after fertilization with NaCNS in Ca-free sea water and with Ca-free sea water alone.

forms developed into exogastrulae with enlarged entoderm and grossly inhibited, radially symmetrical ectoderm (Fig. 31). Often such exogastrulae were stuck together and intimately fused by their entoderms (Fig. 32).

Fertilized eggs submitted to Ca-free sea water alone for 4 hours developed into normal plutei on return to normal sea water. Eight hours' exposure to Ca-free sea water gave blastulae which were slightly irregular in shape but which developed, for the most part, into normal plutei, although in some the oral lobe was slightly inhibited. Ten and one-half hours' initial exposure to Ca-free sea water had, however, a marked effect. When removed to normal sea water, the forms appeared as broken and ruptured middle blas-

tulae showing loss of cells from all surfaces. They developed into bottom forms with inhibited oral lobes and a surplus amount of skeleton and mesenchyme. Anal arms were often rudimentary or absent but, when present, were usually at aberrant angles, askew, asymmetrical, or duplicated (Figs. 33 and 34). Many of these forms stuck together, creating a mass of skeleton and arms. Few, if any, exogastrulae appeared with this treatment.

DISCUSSION

From these experiments it was quite evident that NaCNS, under the conditions used, behaved as an inhibiting agent. This was shown by the retardation in cleavage rate and the inhibited type of larvae that resulted from continuous exposure. No measurements of respiratory rate were made. Apparently the calcium of normal sea water antagonizes, to some extent, the penetration of NaCNS into the cells. When calcium is removed, it seems clear that the agent not only has a greater effect but is much more rapid in its action.

The ectodermized forms produced here in the sand-dollar larva were less extreme than many of those produced in the sea urchin by Lindahl (1933) and Hörstadius (1935) with NaCNS treatment and the experimental isolation of animal blastomeres but did show more or less "animalization." This same agent, however, when properly applied after fertilization, definitely inhibits ectoderm, and most of the larvae develop toward entoderm and mesenchyme. The production of such different larvae with exposure to the same agent before and after fertilization presents an interesting problem.

Lindahl (1936) maintains that before fertilization NaCNS acts directly in increasing or extending the animal gradient of the egg. He fails to consider, however, the possibility that such ectodermization or animalization may be a secondary effect or recovery on return to normal sea water and of the intense activation following fertilization. In this connection it cannot be emphasized too strongly that in our experiments thiocyanate preceding fertilization retarded cleavage and that, at best, only part of the eggs developed into highly ectodermized forms, the maximum being 30 to 40 per cent. If ectodermization or animalization were a direct effect of NaCNS, we should expect that with increase in concentration or exposure period above that at which ectodermization appeared the percentage frequency would increase to approximately 100 per cent, as in exogastrulation by LiCl and various other agents. This does not happen, however. The maximum frequency of ectodermized forms occurs with concentrations and exposure periods not much above those producing little effect, and with further increase ectodermization is progressively replaced by differential inhibition. In view of this evidence the possibility that ectodermization results from recovery, rather than as a direct effect of the thiocyanate when the eggs are treated before fertilization, appears highly probable.

Exposure of unfertilized eggs to NaCNS in Ca-free sea water apparently results in a general depression or slowing-down of physiological processes, the apical or more active region being retarded to a greater extent than the less active vegetal region. Such differential inhibition may be sufficient to decrease the apical dominance to such a degree that the axiate pattern is partly obliterated. With the stimulation to increased activity, resulting from return to normal sea water and fertilization, the lower levels of the gradient, being wholly or partly physiologically isolated, may recover and develop in the same, or almost the same, way as higher levels. In other words, the whole egg may now develop in a manner similar to the original apical portion. Since the apical region normal-

ly gives rise to ectoderm and ectodermal derivatives, the whole egg may now give rise to ectoderm alone, with neither mesenchyme nor gut developing. Such a reconstitution results from an increased scale of organization (see Rulon and Child, 1937), which reaches its peak in forms with a large and centrally placed stomodaeum.

The treatment of fertilized eggs with NaCNS for certain intervals and with certain concentrations results in forms (chiefly exogastrulae) which are not very different from those produced with lithium and other agents (Child, 1940) and lend themselves to a similar interpretation. When the early cleavage stages are exposed to NaCNS, the more active animal pole seems to be primarily inhibited to a greater extent than the vegetal region. The lowered dominance, together with a direct inhibiting action of the agent, may be factors which permit cells to escape from the vegetal region with greater facility than in normal development. Exogastrulae with an extremely reduced ectoderm and enlarged entoderm are not entirely the result of an evagination of the original entoderm. As in the case of Child's lithium-treated larvae, the original entoderm may be partly or completely broken down into mesenchyme-like cells which are lost to the outside or passed into the blastocoel. The large entoderm present may be the result of the inhibiting action of the agent which has depressed the basal levels of the prospective ectoderm to the physiological level of prospective entoderm. Such entodermized ectoderm may be considerably increased in size as a result of recovery on return to normal sea water. Although Child (1940) obtained as high as 100 per cent exogastrulae with lithium treatment, he also noted that Janus green and even crowding tended to entodermize the larvae of certain echinoderms. This, together with the data showing extreme entodermization with NaCNS, does not support the view of the Runnström group that lithium has a specific entodermizing action.

If the exposure time is short or the concentration of the agent is low, there may be considerable recovery and reconstitution of the ectoderm. In many cases the pattern of ectodermal development following initial treatment is considerably distorted. This is particularly true if NaCNS is used in Ca-free sea water solution, although proper exposure to Ca-free sea water alone produces distortion in gastrulated larvae. In general, the oral lobe is markedly inhibited, and excess mesenchyme is present. Commonly the anal arms are at various angles, asymmetrical or askew. Larvae may develop one or many anal arms. These various aberrant forms cannot easily be interpreted, although undoubtedly they represent varying degrees of differential inhibition and recovery of the early embryonic pattern. As stated above, many cells are liberated into the blastocoel during or shortly following the exposure to NaCNS. Calcium-free sea water has long been known to permit the escape or separation of cells in the cleavage or blastula stages. If the treatment is severe, those cells liberated into the blastocoel may develop very little or even cytolyze. However, if the treatment is not too severe, those internal cells may form skeleton in abundance. The appearance of a distorted pattern in anal-arm formation may be related to anomalous distribution or mesenchymal (skeletal) elements, as well as to certain incidental factors, such as contact with the substratum or nearness of other larvae, which may affect the extremely labile condition of the pattern at the critical period of recovery.

SUMMARY

1. Treatment of unfertilized eggs of *Dendraster excentricus* with 20 cc. of 0.54 M NaCNS plus 100 cc. of Ca-free sea water for 12 hours, followed by return to normal sea

water and fertilization, results in retarded cleavage and larvae ranging from ectodermized forms (30–40 per cent) without mesenchyme, gut, or pigment to grossly inhibited forms with excessive mesenchyme, short anal arms, and undifferentiated oral lobes. Higher concentrations and longer exposure periods to this agent decrease the number of ectodermized, and increase the number of inhibited, larvae. Fewer ectodermized and inhibited forms result from lower concentrations and exposure periods. Such evidence favors the view that the ectodermized forms produced by NaCNS treatment before fertilization result from recovery following a differential inhibition of the normal axiate pattern.

2. Fertilized eggs treated with 20–30 cc. of 0.54 M NaCNS plus 100 cc. of normal sea water for 24 hours following fertilization and then returned to normal sea water develop into exogastrulae with considerably enlarged entoderm, reduced ectoderm, and excessive mesenchyme in from 65 to 95 per cent of the cases. In these experiments entodermization seems to result from the depression of the more basal levels of prospective ectoderm to the physiological level of prospective entoderm. Such entodermized ectoderm may be considerably increased in size as a result of recovery on return to normal sea water. The increase in what appears to be mesenchyme seems to result largely or wholly from dissociation and passage into the blastocoel of cells from the original entoderm.

3. With varying concentrations and exposure periods fertilized eggs treated with NaCNS in Ca-free sea water and with Ca-free sea water alone may give rise to larvae ranging from cell-filled blastulae to forms with excessive mesenchyme, with inhibited oral lobe, and with anal arms duplicated, asymmetrical or askew. Such aberrant larvae are interpreted as resulting from varying degrees of differential inhibition and recovery of the early embryonic pattern. Distortion of the pattern may be due to such factors as contact with the substratum, anomalous distribution of skeletal elements, or nearness of other larvae.

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EFFECTS OF MODIFIED RINGER SOLUTIONS ON HEAD RECONSTITUTION IN DUGESIA (=EUPLANARIA) DOROTOCEPHALA[1]

(Twenty-eight figures)

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IN AN earlier paper on a study of *Dugesia* (=*Euplanaria*) *dorotocephala* with Murray's modified Ringer solution (Watanabe, 1937) it was shown that definite and orderly variations in death frequency of pieces along the axis of body indicate a physiological differential or gradient of susceptibility to lethal action of the solution as with many other agents the disintegration processes in whole animals do. The present paper includes the results of experiments on head reconstitution and is chiefly concerned with the effect of various concentrations of the solution upon the gradient of head frequency. As to the head-frequency gradient in this species, the paper by Child and Watanabe (1935) may serve, to some extent, as a general background for this paper.

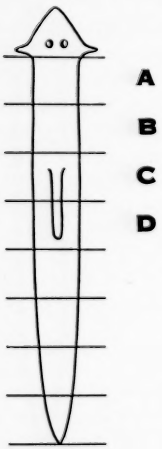

FIG. 1.—Body-levels of pieces used in experiments; each piece is one-eighth of postcephalic length.

The material consisted chiefly of four successive pieces one-eighth of the total postcephalic body length of worms 16–18 mm. in length and representing approximately the anterior zooid. The one-eighth pieces used were designated *A*, *B*, *C*, and *D*, the order being from anterior to posterior levels, as shown in Figure 1. As has often been noted (Child and Watanabe, 1935; Rulon, 1936; etc.), head frequency in reconstitution differs more or less in worms from different localities as well as at different seasons of the year, although it seems not persistently different. Such difference apparently results from difference in environment in nature prior to experimentation. For each series of experiments worms of single collections from single localities were employed.

In order that the animals might be as nearly as possible in the same physiological condition, they were subjected to laboratory conditions with feeding and change of water for at least 2 weeks and then starved 1 week before use, except the worms used in Series 5 and 6. The worms for these series were not fed at all, since, after a trial feeding, they were found to be markedly poor in general condition. The tempera-

[1] The data presented here, like those in the earlier paper (Child and Watanabe, 1935), were obtained during the years of 1932–34 at the Department of Zoölogy of the University of Chicago.

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ture of the laboratory, under which all the experiments described below were performed, was adjusted by thermostat at 19°–21° C.

The solutions and exposure procedure employed were essentially those of the earlier work (Watanabe, 1937). The standard concentration of modified Ringer solution was made up as used by Murray (1928) in planarian tissue-culture experiments and is believed to be approximately isotonic with planarian tissue.[2]

Concentrations used in experiments are given as fractions or multiples of this standard concentration (S) taken as 1. To attain pH 7.6, sodium bicarbonate was added to the solutions of a given concentration; but no attempt was made to buffer the solutions, because any buffer employed, even if not injurious, might introduce effects of the buffer substance. Concentrations and periods of exposure employed are stated in connection with particular experiments.

As suggested earlier (Watanabe, 1937, p. 180), physiological condition of pieces or whole animals in the solutions differs greatly with difference in treatment and depends largely on the relative amount of the solutions to the volume of the tissues and on the frequency of renewal of the solutions during exposure. For this reason a uniform procedure was adopted in all experiments: After section in water pieces were rinsed three times with the solution to be used and 20 or 25 pieces from the same level were put together in 200 or 250 cc. of the solution, i.e., 10 cc. for each piece, without renewal during exposure (see Watanabe, 1937, p. 181). After exposure and three washings pieces remained in water approximately 15 days before head forms were recorded.

The planarian head forms classified by Child (1921)—normal, teratophthalmic, teratomorphic, anophthalmic, and acephalic—constitute a graded series and a convenient basis for distinction of the different degrees of head reconstitution. They have been so often described and figured that further description is unnecessary here (see Child and Watanabe, 1935, and literature cited there). As regards normal heads, however, one point is to be noted. Although the normal head in nature is triangular with two eyespots and two lateral lobes or auricles, in this paper heads with two symmetrical eyespots are recorded as normal, even though shape of head is not entirely normal, in consequence of secondary modifications. The "head-frequency index," a numerical value indicating degree of head development in a given lot of pieces from a particular level, was determined by the formula previously used.[3]

[2] The standard concentration is as follows:

NaCl	2.5 gm.
$CaCl_2+2H_2O$	0.25
KCl	0.05
Distilled water to 1,000 cc.	

[3] Head-frequency index $= \frac{(100n_5 + 80n_4 + 60n_3 + 40n_2 + 20n_2 + 0n_0)}{N}$. In this formula $n_{-5}n_1$ represent the numbers of pieces which develop normal, teratophthalmic, teratomorphic, anophthalmic and acephalic forms, respectively; and n_0, the number of dead pieces. The total number of pieces tested in the lot or series of lots is indicated by N. The numbers 100, 80, 60, 40, and 20 are arbitrary numerical coefficients assigned to the different head forms. As has been pointed out (Watanabe, 1935*b*, p. 136), dead pieces (n_0) are included in this formula because under certain conditions frequency of deaths preceding reconstitution shows a differential distribution in relation to body-level, and it appears desirable to include the total number of pieces in the record. However, definite information concerning head frequency can be obtained only with experimental conditions that give few or no deaths. With high percentages of deaths, head-frequency indices cannot be obtained. The data on head frequency in this paper

HEAD FREQUENCIES OF NORMAL WORMS IN RINGER CONCENTRATIONS ABOVE AND BELOW STANDARD

In *D. dorotocephala* head frequency at a given body-level decreases with decrease in length of pieces; and in pieces below a certain length the frequency decreases from anterior to posterior level of the anterior zooid. In one-eighth pieces head frequency decreases from almost 100 in *A* to a low level in *D*. But, under experimental conditions, these head frequencies are altered in a definite way, with axial differentials. The following data were obtained with an agent not previously used for this purpose and provide further evidence for the orderly relations between concentrations or intensities of agents and the head-frequency gradient.

Series 1. Head frequencies with 14-day exposure to concentrations above standard.—Concentrations from 1 S to 2 S, with controls in well water, were used. In concentrations above 1.9 S total deaths exceed 5 per cent at each level.

As shown by the graphed data of Figure 2, head frequency increases with increase in concentration at each level, except at the *A*-level, at which it is nearly 100 in 1.0–1.8 S and in well water. The increase in head frequency at 1.8 S solution (*P*, Fig. 2) is greatest at *D*-level, next greatest at *C*-level, and least at *B*-level. In consequence of this great increase in head frequency at levels *C* and *D* the gradient of head frequency in 1.8 S solution is distinctly less steep than in 1.0 S or in the controls. In higher concentrations an axial differential in total deaths appears, decreasing posteriorly from the *A*-pieces. This is similar to the decrease in susceptibility of intact animals to many gradually lethal agents.

Series 2. Head frequencies with short-time exposures to concentrations above standard.—Concentrations used were 2.5 S and 3.0 S. In the experiment with 3.0 S solution the pieces were exposed 0, $1\frac{1}{2}$, 3, $4\frac{1}{2}$, and 6 hours, respectively.

Figure 3 shows that head frequency at each level increases with increase in period of exposure up to 3 hours, most in *D*-pieces, very slightly in *A*-pieces. With 3 hours' exposure no deaths occur, but with longer exposures deaths exceed 5 per cent and clearly show an anteroposterior differential. With 6 hours' exposure there were many deaths, most of them without head reconstitution within 48 hours after exposure.

Similar results were obtained with 2.5 S solution. Up to 9 hours' exposure, head frequency in *A*-pieces shows very slight decrease; but in *B*-, *C*-, and *D*-pieces it increases with increase in period of exposure. The 2.5 S solution is much less lethal than the 3.0 S. With $13\frac{1}{2}$ hours' exposure deaths appear in *A*- to *C*-pieces, while in *D*-pieces no deaths occur. With 18 and 27 hours' exposure total deaths for each level exceed 15 per cent and show a beautiful anteroposterior differential.

Series 3. Effect of 14-day exposure to concentrations below standard.—Concentrations used were 0.01 S, 0.05 S, 0.1 S, 0.2 S, 0.4 S, 0.6 S, 0.8 S, and 1.0 S, with controls in well

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were obtained from experiments in which total deaths were less than 5 per cent. With so few deaths, the index, whether based on total number or on living pieces, is practically the same.

It has been pointed out in earlier papers that this formula provides merely a rough and simple means of comparing head frequencies in relation to body-level, length of piece, and environmental conditions and is not based on statistical methods. However, the graphs resulting from application of statistical methods to planarian head frequencies by W. A. Castle (1940) are in general almost identical with those plotted from head-frequency indices. In fact, Castle points out that the method of indices appears in most cases to be entirely adequate for distinguishing differences in head frequency which are not obscure. It has, of course, not been used for any other purpose.

water. Results are graphed in Figure 4. Head frequencies at the concentrations below 0.2 S are not given in this figure. Total deaths in these solutions were more than 5 per cent.

As shown in Figure 4, head frequency at 0.4–1.0 S solutions is always highest in *A*-pieces and decreases posteriorly, and in general it increases as concentration decreases. The difference in head frequency between 0.4 S (*N*) and 1.0 S (*K*) is greatest in *D*-pieces, next greatest in *C*-pieces. less in *B*-pieces, and least in *A*-pieces.

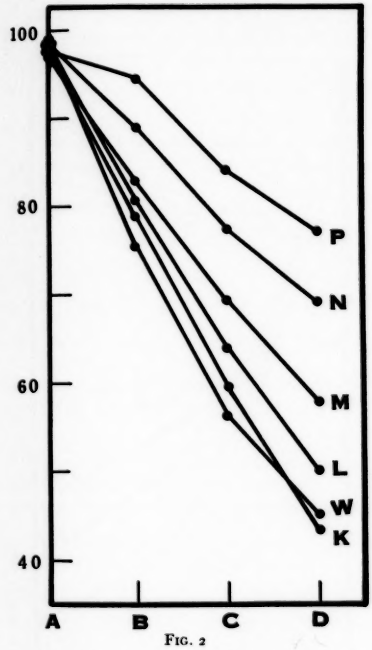


FIG. 2

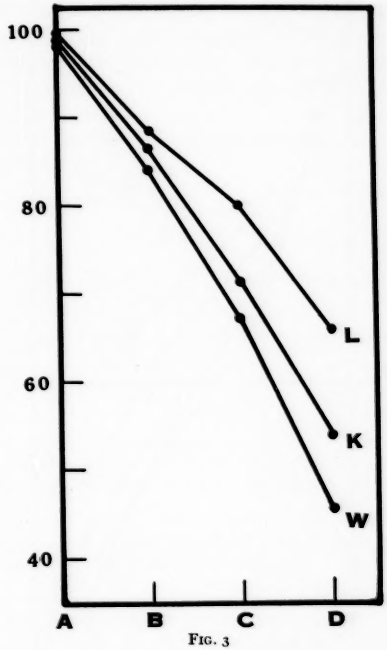


FIG. 3

FIG. 2.—Head frequencies of one-eighth pieces with 14 days' exposure to standard and higher concentrations. *W*, control in water; *K*, 1 S; *L*, 1.2 S; *M*, 1.4 S; *N*, 1.6 S; *P*, 1.8 S. Ordinates are head-frequency indices; abscissae, body-levels. Data are averages of 5 lots, each of pieces from 20 worms for each concentration.

FIG. 3.—Head frequencies of one-eighth pieces in 3 S concentration with different short exposure periods. *W*, control, water; *K*, 1½ hours; *L*, 3 hours. Data are averages for 5 lots, each consisting of 20 worms for each period of exposure.

Series 4. Effects of short-time exposures to concentrations below standard and to distilled water.—Solutions 0.05 and 0.1 S and distilled water were used in this series. To 0.05 S solution the pieces were exposed 0, 9, 18, and 27 hours, respectively; and to 0.1 S solution the pieces were exposed 0, 12, 24, 48, and 72 hours, respectively. Total deaths did not exceed 5 per cent at any level in 0–18 hours' exposures to 0.05 S; and in 0–24 hours' exposures, to 0.1 S. In both concentrations results were very similar as regards relation of

head frequency to level and steepness of the gradient curve to exposure period. Figure 5 represents the results of exposure to 0.05 S. The slight differences in *A*- and *B*-pieces are of doubtful significance, but head frequency increases in *C* and still more in *D* with increase in exposure period. Distilled water was also tested as the extreme of dilution. To bring it to pH 7.6 a minute quantity of sodium bicarbonate was added, as in preceding experiments. With 6 hours' and longer exposures all pieces died, and with 3 hours' exposure so many died that the data have little or no value.

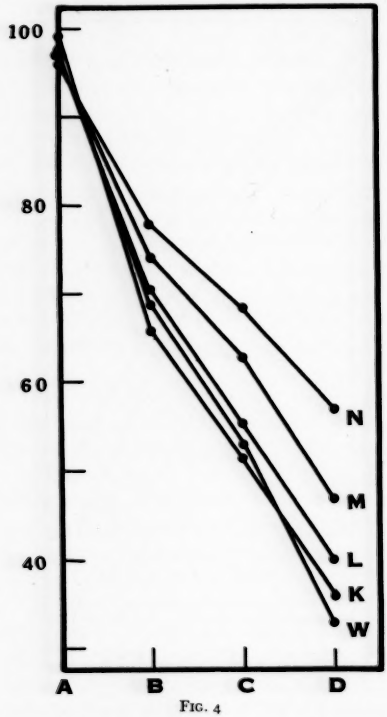


FIG. 4

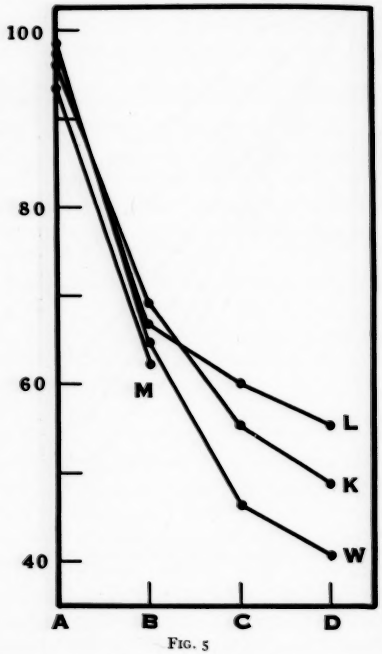


FIG. 5

FIG. 4.—Head frequencies of one-eighth pieces with 14 days' exposure to standard and lower concentrations. *W*, control, water; *K*, 1 S; *L*, 0.8 S; *M*, 0.6 S; *N*, 0.4 S. Data are averages for 5 lots, each of pieces from 20 worms for each concentration.

FIG. 5.—Head frequencies of one-eighth pieces with short exposures to 0.05 S. *W*, control, water; *K*, 9 hours; *L*, 18 hours; *M*, 27 hours. Data are averages for 3 lots of 25 pieces for each level for each period of exposure.

ANOMALOUS HEAD FREQUENCIES OF CERTAIN STOCKS

In certain stocks of worms from Rockford, Illinois, collected in winter and early spring of 1934, an extraordinarily high head frequency and a high bipolar frequency were observed. Head frequencies in normal and some experimental environments are given below. Data on bipolar heads will be reported in a later paper.

The worms of these stocks were evidently not in normal condition: many individuals disintegrated under laboratory conditions, especially after feeding, and pieces showed exceedingly high susceptibility to Ringer concentrations above standard. But these anomalies represented a temporary condition, because the head frequency, biaxial frequency, and susceptibility in worms of other collections from the same spring-fed stream were usually almost the same as those in worms from other localities.

Series 5. Head frequencies of abnormal worms in 14-day exposure to concentrations above standard.—Figure 6 shows the results with 14 days' exposure to 1.0–1.8 S concentrations. As the graphed data show, the head frequencies of the controls, W, are exceedingly high in this series as compared with those in the preceding series: they are all nearly 100. Even in *D*-pieces the index is 92.5. In Ringer, head frequency does not increase but decreases almost uniformly at all levels with increase in concentration. In 1.9 S solution

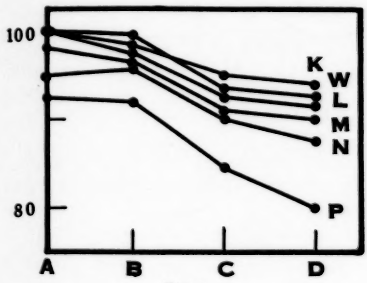


FIG. 6

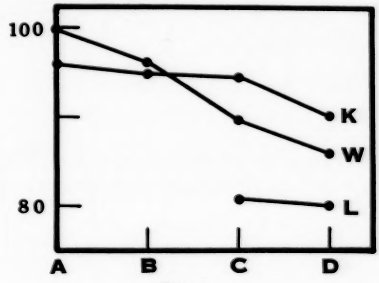


FIG. 7

FIG. 6.—Head frequencies of one-eighth pieces from abnormal worms with 14 days' exposure to various concentrations. *W*, control, water; *K*, 1 S; *L*, 1.2 S; *M*, 1.4 S; *N*, 1.6 S; *P*, 1.8 S. Data are averages of 2 lots of 20 worms for each concentration.

FIG. 7.—Head frequencies of one-eighth pieces from abnormal worms in 2.5 S. *W*, control, water; *K*, 3 hours; *L*, 6 hours. Data are averages of 2 lots of 25 worms for each period of exposure.

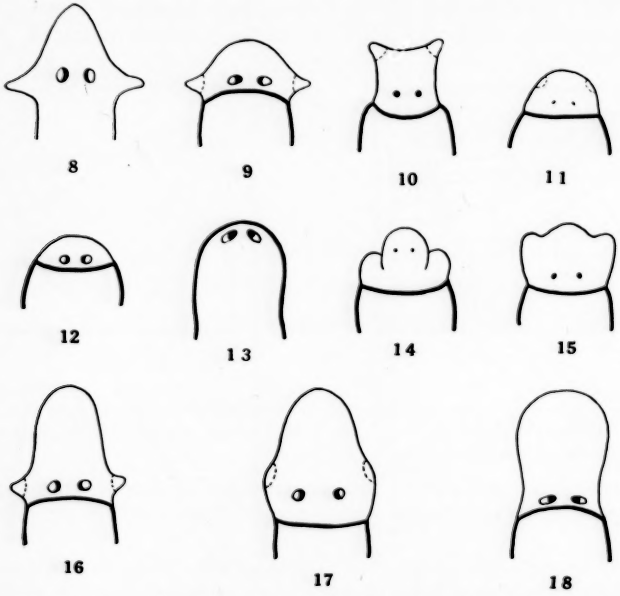
the total death frequency in pieces from this stock was two to three times that in the pieces from the corresponding levels of worms used in Series 1; and in 2.0 S solution all pieces died in this stock, while in Series 1, from 49 to 82 pieces out of 100 were still alive after 14 days' exposure.

Series 6. Head frequencies of abnormal worms in short-time exposures to concentrations above standard.—In this series 2.5 S and 3.0 S solutions were used. General relations of exposure period and head frequencies of the pieces from different levels were nearly the same in both solutions, so that the results with 2.5 S solution only are shown in graphs in Figure 7.

Head frequency of this stock in well water (0 hours' exposure) is still abnormally high, although it is generally a little lower than in the preceding Series 5. The frequency in *C*- and *D*-pieces increases slightly with 3 hours' exposure (*K*) but again decreases at 6 hours' exposure (*L*). At 6 hours' exposure some pieces from *A*- and *B*-levels died, but in *C*- and *D*-levels no deaths occurred.

MEDIOLATERAL DIFFERENTIAL MODIFICATIONS OF NORMAL AND TERATOPHTHALMIC HEADS IN CONCENTRATIONS ABOVE STANDARD

It has been noted in various papers by Child (1916, 1920, 1921) that the different forms of head in regulatory reconstitution of *D. dorotocephala* represent different degrees of differential modification along the mediolateral axis. Similar mediolateral differential modifications of normal and teratophthalmic heads occur with an exposure to Murray-Ringer concentrations above standard. Results of observations are appended here, and characteristic features in modification are shown in semidiagrammatic figures drawn from the observed cases.

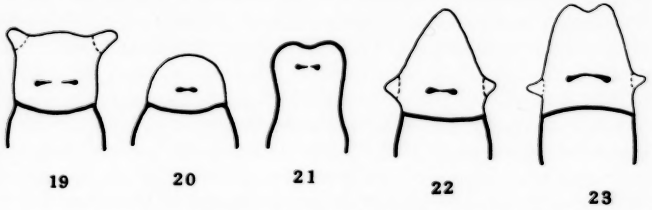


FIGS. 8–18.—Modifications of head form in concentrations above standard. Fig. 8, normal head in nature; Figs. 9–13, mediolateral differential inhibition; Figs. 14–18, mediolateral differential conditioning.

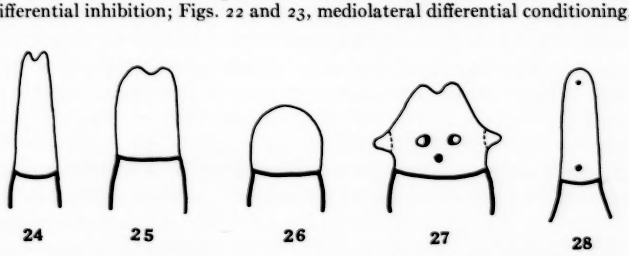
Inhibitory modifications in "normal" heads.—These heads are regarded as normal because eyespots, and presumably ganglia, are normally localized; but other parts of the head may be differentially modified, as compared with the characteristic head form of the species (Fig. 8). With 14 days' exposure to concentrations from 1.9 to 2.2 S the median part of the preganglionic region is more or less inhibited. The preocular length is decreased, and the new head becomes semicircular in shape with two lateral lobes (Fig. 9). With further inhibition the lateral lobes approximate the median line, as in ordinary teratomorphic heads (Fig. 10), and their development is often more or less inhibited (Fig. 11). In extreme inhibition the lobes are entirely absent, and the head is a rounded mass of tissue with two eyespots (Fig. 12). In the higher concentrations, 2.0 and 2.2 S,

regeneration of new tissue may be decreased or almost absent, and eyespots develop in the old tissue, as in Figure 13, a *D*-piece in 2.0 S concentration. In these pieces in water, eyespots develop at some distance anterior to the boundary between old and new tissues.

Secondary modifications of "normal" heads due to differential conditioning.—Figures 14–18 show another interesting feature of differential modification in outline of the head in concentrations above standard. In 1.9 and 2.0 S solutions in some pieces no outgrowth occurs during the first week, then after 10–12 days' exposure a new small mass of tissue with two eyespots develops at the median part of the anterior cut surface, and soon after that another small outgrowth appears on each side of this median one (Fig. 14). This form of head apparently represents a mediolateral differential conditioning in reconstitu-



FIGS. 19–23.—Modifications of teratophthalmic head in concentrations above standard. Figs. 20–22, mediolateral differential inhibition; Figs. 22 and 23, mediolateral differential conditioning.



FIGS. 24–28.—Secondary modifications of head forms. Figs. 24–26, anophthalmic heads; Figs. 27 and 28, secondarily modified teratomorphic heads.

tion. The head in Figure 15 is probably also a result of differential conditioning. In concentration 1.9 S these secondary modifications appear less frequently and are less modified than in concentrations 1.6–1.8 S, in which they are often extreme. In some cases the preganglionic region becomes relatively very large and much elongated. In concentrations 1.6 and 1.7 S this overdevelopment may occur without much inhibition of lateral parts (Fig. 16), but in 1.8–1.9 S there is more or less lateral inhibition (Figs. 17 and 18).

Differential modifications of teratophthalmic and other heads.—In development of teratophthalmic heads in concentrations above standard two opposed types of mediolateral differential modification appear. With higher concentrations, 1.9 and 2.0 S, inhibitory modifications occur (Figs. 19–21), while with lower concentrations, 1.6 S, differentially conditioned forms develop (Figs. 22 and 23).

In concentrations 1.9 and 2.0 S the new outgrowth is not infrequently without eyespots and different in form from the anophthalmic heads developing in water (Figs. 24–26). These probably involve some differential conditioning.

In 1.6 S, secondary transformations of teratomorphic heads occur frequently, even within 2 weeks after section. Figure 27 shows a transformed head without obliteration of a primary median eyespot. The head form of Figure 28 evidently results from secondary median elongation of the anterior outgrowth with a second median eyespot far anterior to the first. Both of these cases undoubtedly represent a differential conditioning in developing teratomorphic heads.

DISCUSSION

It has been shown repeatedly that various inhibiting agents increase planarian head frequency, being most effective on pieces from more posterior levels of the anterior zooid, and that a single concentration of a single agent may decrease head frequency in anterior, and increase it in posterior, pieces.[4] Effects on head frequency of delay of anterior and posterior section (Child and Watanabe, 1935; Watanabe, 1935*b*) have made it evident that the factor inhibiting head reconstitution results from the activation following section at the posterior end of the piece. This inhibiting factor is effective only below a certain length of pieces, which increases from anterior to posterior levels in the anterior zooid and decreases again in the posterior zooid. Moreover, that the inhibiting factor is chiefly or wholly nervous is indicated by the fact that section of nerve cords is almost as effective in increasing head frequency as section of the whole body and by the effectiveness of anesthetics in increasing head frequency (Buchanan, 1922; Watanabe, 1935*b*). There are then two antagonistic factors in determining head frequency in planarian pieces below a certain length—the activity of the cells directly concerned in head development and the effectiveness of the factor inhibiting this activation.

The relation of these two factors differs at different body-levels. At anterior levels the inhibiting factor is effective only in extremely short pieces; consequently, in the pieces ordinarily used for experiment, one-sixth to one-eighth of total postcephalic length of 16–18 mm., anterior pieces have a high head frequency, and any effect of an inhibiting agent will decrease it by action on the cells concerned in head regeneration. In posterior pieces, however, where the factor inhibiting head development is much more effective, an inhibiting agent may inhibit this factor more than it inhibits the head-forming cells and so may increase head frequency; or, since the inhibiting factor is temporary in action, it may be abolished by a short exposure to the inhibiting agent and, after return to water, head development can proceed without inhibition. The various possibilities have been realized in experiment.

It is evident that the planarian Ringer solution acts like an agent inhibiting the inhibiting factor and so increasing head frequency, the amount of increase decreasing from posterior to anterior pieces. Since the solutions have no appreciable effect on head frequency in anterior pieces, they evidently do not greatly inhibit the developmental activities concerned in head regeneration, though they do bring about some degree of differential modification of head form—either differential inhibition or secondary differential conditioning or acclimation.

In the higher Ringer concentrations, intact animals soon become incapable of co-ordinated locomotion or are more or less paralyzed, though muscular contraction is possible. This suggests interference with nervous activity and indicates why this agent is so con-

[4] Child, 1916, KCN; Buchanan, 1922, anesthetics; Hinrichs, 1924, caffein; Rulon, 1936, 1937, CO_2, H-ion, organic acids; Miller, 1937, strychnine.

sistently effective in increasing head frequency in the more posterior pieces and has no significant inhibiting action on head frequency in anterior pieces. With long-time exposures to concentrations above standard the increase in head frequency increases with concentration up to beginning of lethal action (Fig. 2). With short exposures it increases with exposure period (Fig. 3).

However, with long exposures to concentrations below standard the increase in head frequency increases with decrease in concentration (Fig. 4), and with short-time exposures, with increase in exposure period (Fig. 5), and distilled water is lethal. In these lower concentrations, as in the high, there is no significant decrease of head frequency in anterior pieces, that is, the low concentrations have little or no inhibiting effect on the regenerating tissue. Presumably, therefore, they increase head frequency by inhibiting the inhibiting factor, like the high concentrations; but it appears probable that the high and low concentrations accomplish this result by changes in opposite directions of the electrolyte balance.

In normal worms the variations in head frequency are usually limited within a certain range characteristic for each species.[5] However, head frequency of pieces varies greatly with nutritive condition, physiological age, and environmental conditions in nature prior to experimentation. As shown in the preceding pages, head frequencies of certain worms were extremely high at all levels with very slight gradient. Similar decrease in gradient has also been found in rate of head development in a Japanese planarian. In normal worms of this species, like *D. dorotocephala* and other planarians,[6] the rate decreases from anterior to posterior pieces, but in a certain stock it was found nearly equal at all levels of body (Watanabe, unpublished data).

Reaction of the pieces from all levels of the aberrant stock to Ringer concentrations above the standard resembles that of the most anterior pieces from normal worms in showing no increase in head frequency, like that in pieces from lower levels of normal worms; their head frequencies decrease rather continuously, but slightly, with increase in concentration or exposure time. In these forms with high head frequency the factor inhibiting head development is almost absent, and the effect of the Ringer concentrations is almost entirely on the regenerating tissue; consequently, it decreases head frequency, but, since its effect on this tissue is slight, as shown by Figures 2–5, the decrease is slight.

The mediolateral modifications of head form appear to be either direct mediolateral differential inhibitions or secondary modifications due to differential conditioning similar to those obtained by Child (1921) with ether and alcohol. In the differential inhibitions the median region is most inhibited; in the secondary modifications of differential conditioning it is at first most inhibited, but later it shows relative or absolute overdevelopment. With Ringer these modifications concern chiefly the preocular region. This probably means that inhibition occurs rather slowly and so affects chiefly the later stages of head development rather than eyespots or ganglia; but it is not extreme, and differential conditioning occurs rather rapidly. This is what might be expected with an agent of this sort and is in line with its general effects on head frequency.

[5] Abeloos, 1930; Sivickis, 1923, 1931; Child and Watanabe, 1935; Watanabe, 1935*b*, 1937, p. 294, footnote; etc.

[6] Watanabe, 1935*a;* Abeloos, 1927, 1930; Sivickis, 1931, 1933; Buchanan, 1933.

SUMMARY

1. Four successive pieces, approximately one-eighth the postcephalic length of animals 16–18 mm. and representing approximately the postcephalic length of the anterior zooid, were used in all experiments.

2. The modified Ringer solution used as agent has no significant effect on head frequency in "standard" concentration, which is believed to be approximately isotonic for planarian tissue.

3. In concentrations above and below standard, head frequency is increased. The increase is greatest at the most posterior level of head development (*D*-pieces), decreases anteriorly from level to level, until at the most anterior level (*A*-pieces) there is no significant increase, and perhaps in some cases a very slight decrease.

4. With long-time exposure (14 days) to concentrations above standard and supposedly hypertonic, head frequency increases with increase in concentration to the beginning of lethal effect. With exposures of a few hours to high concentrations head frequency increases with increase in exposure period.

5. With 14-day exposures to concentrations below standard, supposedly hypotonic, head frequency increases with increasing dilution; but below 0.2 S lethal effect increases with further dilution. With short-time exposures to 0.05 S head frequency increases with increase in exposure period.

6. In general, the modified Ringer acts like other inhibiting agents in increasing head frequency. The experimental evidence indicates that it acts almost entirely by inhibiting the factor, apparently nervous in character, which results from posterior section and inhibits head development. The higher concentrations abolish co-ordinated locomotion and paralyze more or less completely. In the lower concentrations the general effect on head frequency is the same as in the higher, though evidently produced by change in electrolyte balance in the opposite direction.

7. In an aberrant stock showing very high frequency with only slight decrease from anterior to posterior levels, various Ringer concentrations above standard decrease head frequency slightly at all levels. In this material the factor inhibiting head development is almost wholly ineffective, and the slight decrease in head frequency by Ringer represents direct effect on the regenerating tissue.

8. None of the concentrations used have any significant differential effect on the earlier stages of head regeneration but later stages of head regeneration, determining the general form of the head may be differentially modified in two opposite directions in concentrations above standard. In differential inhibition the median preocular region is more inhibited than lateral regions. With secondary modification by differential conditioning this region is relatively or even absolutely overdeveloped in size.

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THE ROLE OF A CUT SURFACE IN TUBULARIA REGENERATION

(Five figures)

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IT IS generally recognized that a recently cut surface is prerequisite to regeneration. This paper tells of an attempt to learn the factors associated with a cut surface which evoke the regeneration of lost structures in *Tubularia crocea*.

The first recent step toward a solution of this problem in *Tubularia* was made by Zwilling (1939). He found that not the cutting of tissue but, rather, an unrestricted contact of tissue with sea water was a necessary precursor to regeneration.

It was suggested that the causal agent associating sea water and regeneration might be oxygen. This suggestion was inspired by the knowledge that the oxygen concentration in the range normal for sea water greatly affects the rate of regeneration of *Tubularia* (Barth, 1938*a*). Furthermore, it is shown in the same paper that when the oxygen tension is high the regenerant is long, extending far back from the cut surface. It was suggested that possibly the length of the regenerant is determined by the length of the effective oxygen diffusion gradient, with the oxygen entering primarily through the perisarc-free, cut surface.

With the facts and ideas presented by Barth and Zwilling as a foundation, an attempt has been made to determine some of the factors important in regeneration and associated with a cut surface.

OXYGEN AND THE CUT SURFACE

The first question asked was whether oxygen may be considered to be the stimulus to regeneration. The common method of preventing regeneration in *Tubularia* is to tie ligatures around the cut ends of the stem. If ligatures prevent regeneration by excluding oxygen, regeneration might be expected to occur when oxygen bubbles are added to the coelenteron and the cut ends are ligatured.

Oxygen and air injections were performed under water in a Petri dish. The injection pipette was a glass tube drawn out to a narrow tip, small enough to be inserted in the coelenteron. The pipette was sealed to the dish with paraffin. After the filling of the coelenteron, ligatures were secured about the stem ends with No. 100 cotton thread.

In order to learn whether the effect of oxygen was a specific chemical effect, the experiment was controlled by one group of stems filled with another gas, nitrogen. Another set of controls was filled with oxygen, but the gas was immediately released. The latter are called "empty." Gas disappeared from the gas-filled stems slowly. Usually a small remnant of the bubble was present at the time for primordium appearance.

The experiments are divided into two groups. The stems of one group were kept in running sea water; and those of the other were left in standing sea water, which was changed twice daily. Each group is subdivided into those kept between 10° and 15° C. and those between 18° and 22° C.

The results are presented in Table 1. It may be seen that approximately 20 per cent of the ligatured stems in the running, warm-water group regenerated when filled with air or oxygen. Without added oxygen the percentage of regeneration was much less. The clear difference between the O_2-filled and the empty stems indicates that the latter suffered from a lack of oxygen. This lack was the result of ligatured ends. It is concluded that the open stem end furnishes passage to an amount of oxygen which makes

TABLE 1

COMPARISON OF NUMBERS OF STEMS FORMING PRIMORDIA UNDER VARIOUS CONDITIONS

(All Stems Ligatured at Both Ends)

Condition	Temperature	Total Number	Regenerating Stems	
			Number	Percentage
	Set A. Running Sea Water			
Air-filled	10°–15°	10	0	0
O_2-filled		26	1	4
N_2-filled		12	0	0
Empty		10	0	0
Air-filled	18°–22°	34	7	21
O_2-filled		48	10	21
N_2-filled		40	1	2.5
Empty		124	3	2.5
	Set B. Still Sea Water			
O_2-filled	10°–15°	8	0	0
N_2-filled		5	0	0
Empty		15	0	0
O_2-filled	18°–22°	20	0	0
N_2-filled		20	0	0
Empty		20	0	0

the difference in some cases between regeneration and no regeneration. The cut surface allows greater passage of oxygen to the tissues than occurs when all entering oxygen must pass through perisarc. Enough oxygen can enter through perisarc to keep stems alive for weeks, but a greater supply than this is necessary for regeneration when there is no passage of substances to and from the stem through an open surface.

A role other than oxygen passage is also played by the cut surface in regeneration. This may be seen from a comparison of the results obtained when O_2-filled stems were kept in running water with the results obtained when stems were kept in standing water. Twenty-one per cent of the O_2-filled stems regenerated in warm running water. In standing water none regenerated. It appears that the exchange of substances between

tissue and water is too slow for regeneration to occur when the water is still. It is fairly certain that the stems had sufficient oxygen. They kept their bubbles for 20 hours or more, and oxygen is necessary for regeneration only during the first 5 or 10 hours of the regeneration period (Barth, 1940*c*). Moreover, 20 stems were reinjected with oxygen when the first bubble had grown small. Only two of these regenerated. This leaves the possibilities that the stems did not regenerate because other substances entered or left the tissue too slowly. The open end, then, is, in most cases, a necessary passageway not only for oxygen but also for other substances whose presence or absence may inhibit regeneration. The possibility that other substances enter too slowly when there is no open surface has not been explored. An argument that regeneration-inhibiting substances build up when there is no open end is presented in another section of this paper.

The next experiment was designed to show whether regeneration can commence without a region open to sea water and consequently without a region more exposed to oxygen than before the cut. Stems were ligatured for a part of the preprimordium period and kept in standing sea water. The ligatures were then removed from the distal

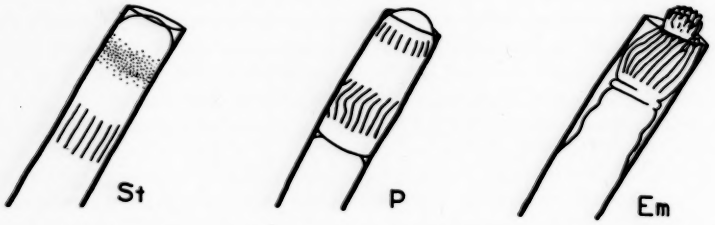


FIG. 1.—Stages in hydranth regeneration at cut end of *Tubularia* stem. *St* = striated stage when a primordium is first visible. The striations are proximal tentacles in the process of formation. The coenosarc has become pink in the region where the distal tentacles will form (indicated by stippling). *P* = pinched stage, when the primordium is partially constricted from the rest of the coenosarc. *Em* = emerging stage when the young hydranth is emerging from the perisarc.

ends. The regeneration times of these stems were compared with those for control stems which had open distal ends from the beginning of the experiment. In order to eliminate the complicating factor of competition by a proximal hydranth, proximal ends were ligatured from the beginning of the experiment on both control and experimental stems. It may be seen from Table 2 that, although distal ends were ligatured for as much as 22 hours, the regenerants which appeared there were only a very few hours behind those appearing in the stems which had been open at the distal end the entire time. Regeneration had started while the stems were ligatured. *Tubularia marina* also starts to regenerate at closed ends when stems are isolated (Morgan and Stevens, 1904). This shows quite strikingly that an increased oxygen supply which results from exposure of an end to sea water is not the stimulus to regeneration. Stems without that exposure to sea water start to regenerate. Distal regions start to regenerate following isolation even without exposed surfaces. An exposed surface is necessary, however, for the continuation of the regeneration. As shown above, one reason for regeneration with an exposed surface is the resulting greater oxygen supply. The experiment just described was conducted in November, and the water temperature was approximately 16° C. Ten times the same experiment was repeated with summer stems at temperatures of 18°–21° C.

These showed no evidence of having started to regenerate during the period of ligature. However, summer stems did begin to regenerate while ligatured when the temperature of the sea water was reduced to 16° C. Possibly not enough oxygen diffuses through the perisarc from the warmer water. Whatever the explanation may be, the fact is established that regeneration is not always dependent for its start on the increase in oxygen supply which follows cutting. The fact that stems do not complete the process

TABLE 2

COMPARISON OF REGENERATION TIMES FOR STEMS LIGATURED FOR VARIOUS LENGTHS OF TIME*

Number of Stems	Hours Ligatured at Distal End	Hours To Reach Stage	
		Pinched	Emerging
5	0	46	55
5	4	46	56
5	20	48	60
5	0	46	55
5	3	46	57
5	22	50	62
10	21	51	62

* The times recorded are hours from the beginning of the experiment when the stems were isolated. The time given to reach a stage is the time for the middle stem of each group to reach that stage. An illustration of these stages will be found in Fig. 1.

All stems were 20 mm. long and were ligatured at the proximal end throughout the experiment.

Conditions: Still water for all stems ligatured distally until distal ligatures were removed from all stems. All the stems were then kept in running water. The stems never ligatured at the distal end were kept in running water from the beginning of the experiment. Temperature was $16° \pm 2°$ C.

of regeneration when the ends are closed brings us again to another factor associated with a cut surface. Before this other factor is considered, the role of oxygen is explored further.

OXYGEN AS A FACTOR DETERMINING THE LOCUS OF REGENERATION

Regeneration occurs at cut surfaces, and the oxygen supply to the tissues at those surfaces is presumably higher than elsewhere. Does a high local oxygen concentration favor regeneration locally?

Observations on the O_2-filled, ligatured stems in the first experiment suggested the possibility that high local concentrations of oxygen favor regeneration. Only those stems which attained the stage where definite primordia were present are included in Table 1. Many more stems—over half—showed an increase in pink pigment after oxygen injection. A few of the N_2-filled stems also became pink. With some of the O_2-filled stems this pink pigment appeared evenly throughout the stem. In others the increase in color was confined to the distal end. A few stems showed pink around small bubbles of gas which remained. Although definite primordia appeared only once in a region other than the distal, it was considered possible that these local pink areas were young regeneration

centers. Some stems were selected which showed localized pink pigment at some point other than the distal. These stems were cut transversely in such a way as to leave the pink portion near the cut surface. The transverse cut was made 24 hours after the oxygen injection and a few hours after the disappearance of the bubble. In a few of these, hydranth primordia appeared in the pink region within 3–10 hours after cutting. Unligatured stems at the temperature of these experiments begin to show primordia approximately 20 hours after cutting. The pink regions may be considered local regeneration centers. It is to be noted that hydranths do not develop in these centers unless the stem is opened.

It is impossible to decide immediately whether oxygen, acting chemically, favors regeneration locally. The oxygen bubble, by blocking the circulation, might have allowed more proximal regions to regenerate. A drop of oil, blocking the coelenteron circulation, has been shown to diminish the dominance of distal over proximal regenerants (Barth, 1938*b*).

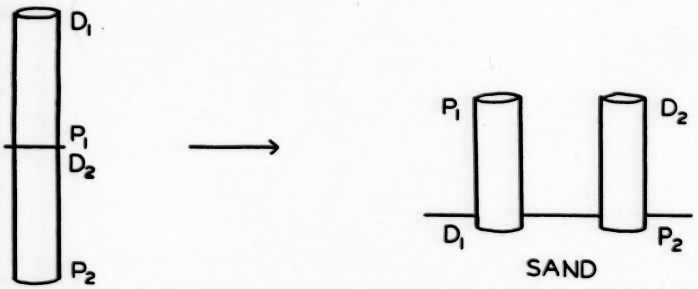


FIG. 2.—The plan for a demonstration of polarity in *Tubularia*. Stem cut into two segments after removal of distal hydranth. The subscript 1 indicates the more distal segment; the subscript 2 indicates the more proximal segment. *D* and *P* denote the distal and proximal ends of the segments. Regeneration at D_1 and P_2 inhibited by placing in sand. Adapted from an experiment by Loeb.

A modification of an old experiment by Loeb furnished a test for the hypothesis that bubbles in the coelenteron diminish the dominance of distal over proximal regions. Loeb (1892) found, when *Tubularia* stems were sectioned as in Figure 2 and regeneration at D_1 and P_2 was inhibited, that regeneration was completed at D_2 much sooner than at P_1. Driesch (1899) has shown that more distal stem levels regenerate faster than do more proximal levels. In Loeb's experiments the regenerant at P_1 was drawing upon more distal levels, D_1–P_1, than was D_2, which drew from the region D_2–P_2; yet regeneration at P_1 was slower than at D_2. The two neighboring regions at P_1 and D_2 must have had a similar constitution with respect to the materials which are the physical basis of the observed activity gradient. One region, P_1, however, was drawing, during reconstitution, upon higher gradient levels; and the other, D_2, upon lower, more proximal gradient levels. It is known (Barth, 1938*b*) that a competition between distal and proximal regenerating regions exists, with the distal being favored. This experiment of Loeb appears to indicate that competition still exists between distal and proximal regions even when the inhibition at the distal end is great enough to prevent the appearance of a regenerant there. Although the D_1 and P_2 regions are both inhibited, they still

affect the rate of regeneration at P_1 and D_2. The competition of D_1 with respect to P_1 is more effective than that of P_2 with respect to D_2.

If oxygen causes nondistal regeneration by blocking the circulation and thereby the dominance, other gases should do the same. In this experiment three groups of stems were selected. The stems of one group were filled with oxygen, those of another with nitrogen, and those of a third were left without added gas. All stems were ligatured at both ends at the beginning of the experment. After varying lengths of time a transverse cut was made, as shown in Figure 3. The time to reach particular stages was recorded for the regenerants at P_1 and at D_2. The results are summarized in Table 3.

The empty stems of Groups A and B regenerated as expected. The regenerants at P_1 formed much more slowly than those at D_2. Presumably the slow rate at P_1 was caused by the competition of more distal regions. When a gas was added to the coelenteron, either oxygen or nitrogen, the inhibition at P_1 was diminished. This shows that internal oxygen bubbles are unsatisfactory when testing for local chemical effects

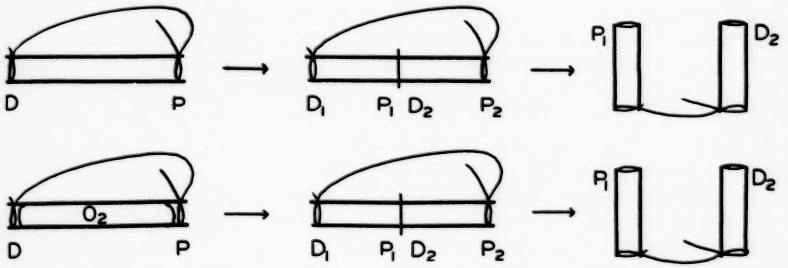


FIG. 3.—Diagram of experiment to test influence of oxygen on competitive behavior of stems. One group of stems ligatured empty, the other group filled with oxygen and ligatured. Both groups cut in middle after oxygen had disappeared from second group. Ligatures at distal and proximal ends connected to allow comparison of regeneration times at D_1 and P_2 in individual cases. Notations as in Fig. 2.

of oxygen, since they have a physical effect, that of blocking the circulation. Another method was devised for use in study of local oxygen effects.

The data of Table 3, although of little value in settling the question whether oxygen is a local stimulant to regeneration, are interesting in other ways. In a few of the cases there was visible evidence that regeneration started in the distal region of empty stems when no cut surface was exposed to oxygen. The fact that P_1 regeneration was extremely slow is considered further evidence that regeneration had started in the more distal regions. Again it is shown that exposure to oxygen cannot be considered the initiating stimulus to regeneration.

The D_1 center, next to the ligature, does not persist as a dominant region. After a cut is made at P_1, a regenerant at P_1 usually dominates, but the effect of D_1 is great enough to retard regeneration at P_1.

None of the stems in Group C of Table 3 give much evidence of the polarity effect. These stems were ligatured for 62 hours before the P_1–D_2 cut was made. Regeneration was more rapid at D_2 in 14 cases, more rapid at P_1 in 7 cases, and the same rate was observed at both D_2 and P_1 in 8 cases. After stems were ligatured for even longer periods—5, 8, 10, 12 days—no difference between the average rates of regeneration at P_1 and D_2

was noticeable. In some stems regeneration was more rapid at D_2; in others a hydranth appeared at P_1 first. The absence of the polarity effect in stems tied off for longer periods is considered an indication that the distal region does not remain an actively competing region when the ends are ligatured. Possibly the fact that almost half of these stems formed buds somewhere between the ligatures while still ligatured is evidence that

TABLE 3

COMPARISON OF REGENERATION TIMES OF DISTAL AND PROXIMAL HYDRANTHS FROM THE SAME STEM LEVEL AFTER INJECTION OF STEM WITH N_2 AND O_2

Group	Conditions	Number of Stems in Group	Treatment	Number of Cases in Which Given Region Regenerated More Rapidly			Region*	Time in Hours for Median Stem of Each Group To Reach Given Stage	
				D_2	P_1	Same Rate at Both Regions		Striated	Emerging
A.....	Standing sea water changed daily; temperature, 19° ± 2° C.; ligatured 26 hours	10	Empty	8	0	2	D_2	43	66
							P_1	66†	89
		10	O_2-filled	7	1	2	D_2	37	54
							P_1	40	61
		10	N_2-filled	6	2	2	D_2	43	49
							P_1	49	70
B.....	Slowly running sea water; temperature, 17° ± 2°; ligatured 48 hours	5	Empty	5	0	0	D_2	.....	42
							P_1	.....	58‡
		5	O_2-filled	0	2	3	D_2	48	58
							P_1	42	56§
C.....	Slowly running sea water; temperature, 20° ± 1°; ligatured 62 hours	10	Empty	5	2	3	D_2	31	41
							P_1	34	44
		10	O_2-filled	4	3	3	D_2	28	36
							P_1	28	35
		9	N_2-filled	5	2	2	D_2	24	34
							P_1	29	42

* Segments lettered as in Fig. 3. Stems 20 mm. in length, ligatured for varying lengths of time, then cut in middle when bubbles were very small or had disappeared.

† Three stems were very pink at D_1 ligatures at 69 hours.

‡ A hydranth formed at the ligatured end D_1 in one case.

§ Oxygen-filled stems showed several regions of concentrated pink along their lengths.

the distal end no longer dominated. The point of appearance of buds was not restricted to any region of the stem. Some arose near the distal end, some near the proximal, and others in the middle region. Although the distal region no longer actively dominates in stems ligatured for long periods, it can be shown that the gradient structure remains. After the ligatures were cut away and both the distal and proximal ends were exposed to sea water, the distal region developed more rapidly than the proximal, and domi-

nance was expressed. These results are similar to those obtained by Peebles (1931) in stems ligatured for several days.

The data of Table 3 may be considered evidence in support of the competition theory of dominance. The gases which block the circulation also decrease the dominance of distal over more proximal regions.

All of the direct demonstrations that dominance is established by way of the circulation have been made by stopping the circulation. The problem has been attacked by another method. This time circulation was permitted, but the tissues between two regions in question were severed. Four groups of stems were prepared. All the stems were approximately 10 mm. in length. The tissue and the circulation between distal and proximal regions were left intact in one group. Stems ligatured in the mid-region comprised a second group. In these both the tissue and the circulation were severed. The stems of a third group had the tissue severed between distal and proximal regions, but circulation continued through the coelenteron between distal and proximal regions. This was done by inserting a short capillary into the coelenteron. The stems were rolled over the bottom of the operating dish, and pressure was applied by forceps in the region of the capillary. This served to force the tissue away from the space between perisarc and capillary. A ligature was then tied around the stem. The ligature prevented reunion of the severed tissue. Circulation, somewhat impaired, persisted for approximately 20 hours through the capillary lumen. It was later blocked by the formation of tissue across the ends of the capillary. The stems of the last group had their circulation blocked by a short glass rod. The tissue was left intact. It may be seen from Table 4 that a distal regenerant usually prevented regeneration in the proximal region when the circulation and the tissue were intact. If both circulation and tissue connections were severed by a ligature as in Group B, regeneration occurred at both distal and proximal ends. Distal regions did not dominate when the tissue was intact but the circulation blocked, as in Group C. Distal regions did dominate when the tissue was severed and only the circulation remained. Therefore dominance is mediated by the coelenteron.

It has been shown that some stems without an open surface do not regenerate because insufficient oxygen is present. An attempt to learn whether local high oxygen concentration plays a part in determining the locus of regeneration was at first unsuccessful. The attempt was unsuccessful because injected oxygen bubbles diminished the dominance of distal regions by blocking the circulation. Local oxygen effects have been studied by other methods.

Torrey (1912) showed that when one half of a stem was bathed with boiled sea water and the other half with boiled and aerated sea water the regenerant always formed at the open end of the half bathed with aerated water, whether that end was distal or proximal. Miller (1937) conducted a similar experiment but, instead, bathed half of the stem in sea water saturated with oxygen and the other half in N_2-saturated sea water. The new hydranth always formed on the O_2-bathed end. In these experiments conducted by Torrey and by Miller the end of the stem at which the hydranth did not form was bathed in water practically free of oxygen. The experiments show that regions in which the oxygen concentration is very low do not regenerate. In the present work we are interested in learning whether the greater oxygen supply to the tissues near a cut surface is sufficient to enable that region to be the dominant regeneration center.

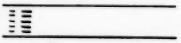
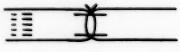
The experiment depicted in Figure 4 entails treatment of cut surfaces in several different ways. Here *a* represents cut pieces of stem used as controls, and *b*, *c*, and *d* show

stems whose ends were covered with large-mouthed caps. Such large-mouthed caps containing an oxygen bubble were placed over the ends of other stems, as indicated in *e–g*. The O_2-filled caps of *h* and *i* differ from those of *e–g* in that their narrow ends fitted closely over the stems. The stems in the *j*-group were ligatured at the distal end and covered with large-mouthed oxygen caps at the proximal end.

In Figure 4 the stems *b–d* differed from the *e–g* stems in the concentration of oxygen present in their caps. In the *b–d* group hydranths did not form at capped ends. In the *e–g* group, capped with oxygen, hydranths formed at the distal end, no matter whether the oxygen was applied distally, proximally, or at both ends. (Sometimes they also formed at the proximal end, but not with significantly greater frequency than in the controls.) Caps locally inhibit hydranth formation. This inhibition is over ridden when

TABLE 4

THE EFFECT ON DOMINANCE OF OBSTRUCTION OF CIRCULATION AND SEVERANCE OF TISSUE

Group	Treatment and Usual Place of Regeneration	Number of Stems	Pinched Primordium at Distal before Any at Proximal	
			Number	Percentage
A.....		30	28	93
B.....		28	3	11
C.....		11	1	9
D.....		30	20	67

oxygen is present in high concentration. Whatever may have been the mode of action of the caps in preventing regeneration at the distal end, their effect was neutralized by the added oxygen. The oxygen in this case acted locally in allowing regeneration at the near-by surface. It is to be noted, however, that oxygen applied proximally did not enable the proximal region to overcome the inherent dominance of the distal region. In fact, the distal regions of the stems treated proximally with oxygen, *f*, regenerated more rapidly than either the uncapped controls, *a*, or the proximally capped stems, *c*. The *j*-stems, which differed by having a distal ligature, formed no distal hydranths. The stems shown in *h* and *i* differed from *e* and *g* only in that the small open ends of their O_2-filled caps fitted closely over the perisarc. When the tight-fitting caps were used, regeneration was inhibited at the capped end, in spite of the fact that the oxygen bubble was present. This result indicates that a factor other than oxygen was responsible for the inhibition at this end, which was bathed with a very small volume of sea water. The inhibition might result from the depletion of substances obtained from sea water and/or from the accumulation of metabolic substances removable by sea water.

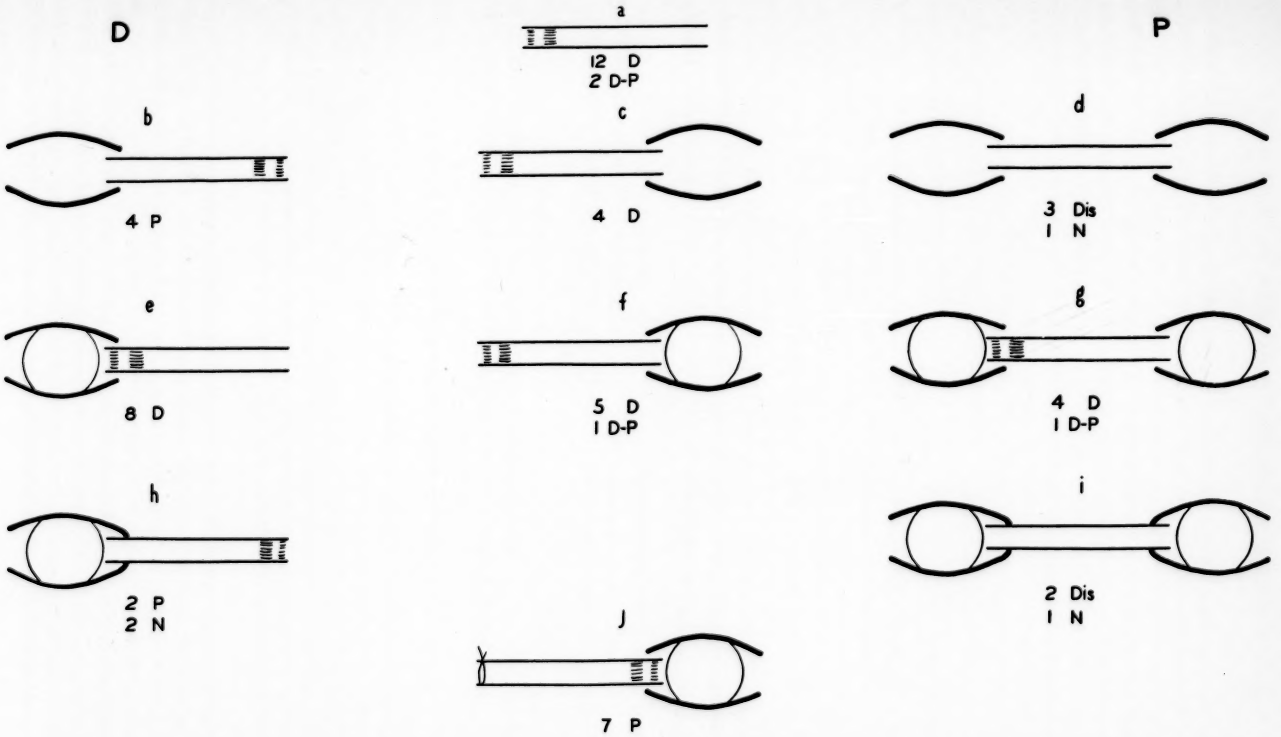


FIG. 4.—Diagram of capping experiments. Position at which regeneration occurred indicated. Distal ends of stems facing left. *D* indicates regeneration at distal end only, *P* indicates regeneration at proximal end only, *D–P* denotes regeneration at both ends. *Dis* = disintegrating coenosarc. *N* = no regeneration. Conditions: Slowly running sea water. Temperature, 20°±0.5°. Stems 20 mm. in length.

The results of the experiment shown in Figure 5 are similar to those just described, but the experiment was conducted in a somewhat different way. If the smaller caps, the Figure 5 type, even though they contained oxygen, were left on stems until reconstitution had occurred, the regenerant never formed at the capped end. In the experiment shown, caps were left in place only 19 hours. The position and the stage of regeneration 31 hours after the caps were removed is recorded. It may be seen that the distal regenerants of the formerly O_2-capped stems (*a*) were in the pinched stage, as were

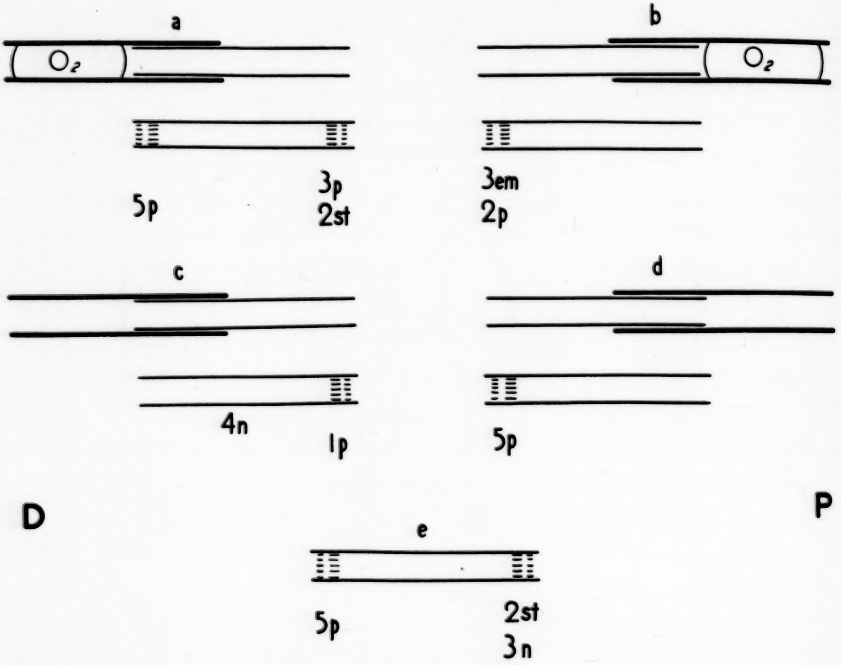


FIG. 5.—Diagram of results of further capping experiments. Stems capped from fourth to twenty-third hour after cutting. The position and stage of regeneration 31 hours after caps were removed is recorded. See Fig. 1 for stages of regeneration. n = no regeneration. Distal ends of stems at left. Conditions: Standing sea water. Temperature, $18.5° \pm 2°$. Stems 25 mm. in length.

those of the untouched controls (*e*). On the other hand, distal regenerants did not appear in the *c*-group, which had been capped but had not received the added oxygen. This is interpreted to mean that a low oxygen supply at one end of a stem in the period before the appearance of primordia inhibits regeneration at that end. There is also the possibility that a cap inhibits during this early period in some way other than by restricting the oxygen supply. If this is the case, the beneficial effect of oxygen can largely neutralize the inhibition of a cap during the early hours of reconstitution. There is some evidence that even the O_2-filled cap inhibits locally, because in *a* of Figure 5 some of the

distal hydranths had not developed so far as those of *b*. Moreover, proximal hydranths formed on the *a*-stems but not on the *b*-stems which had had proximally placed oxygen caps.

Definite evidence of a locally inhibiting factor other than a low oxygen supply comes from the result that regenerants never formed when the small O_2-filled caps were left in place several days.

The group showing the most rapid development at the distal end was, as in the former experiment, the group which had been treated with oxygen at the proximal end. When much oxygen was available to the stem, the distal region regenerated most rapidly, whether the oxygen had entered from the distal or the proximal end.

Since distal regenerants appeared slightly ahead of controls when oxygen was applied proximally in these experiments with few stems, an experiment was performed with more stems to see if more rapid distal regeneration really follows proximal oxygen treatment. One group of 50 stems was capped proximally with oxygen, as in Figure 4, *f*. Another group of 50 was capped proximally without oxygen, as in Figure 4, *c*. Fifty more stems remained uncapped. Here, again, regeneration occurred at the distal end when the oxygen was added at the proximal end. The proximal region even with added oxygen was dominated by the distal end. Furthermore, the rate of regeneration at the distal end was not decreased when oxygen was applied proximally. The average times to reach the pinched-off stage in the three groups were very close. The uncapped took 31.7 hours; the empty-capped, 31.3 hours; and the O_2-capped, 30.8 hours. The averages are so close and the times for individual stems so variable that it is impossible to conclude from this experiment that oxygen applied proximally causes an increased rate of regeneration in the distal region.

It is concluded from the capping experiments that a cap over a cut surface neutralizes the effect of the cut surface by diminishing the local oxygen supply. However, the effect of added oxygen at a proximal cut surface is not great enough to overcome distal dominance. Evidence that another factor associated with a cut surface also plays a part in determining the place of regeneration has continually appeared.

RELEASE OF INHIBITING SUBSTANCES AT THE CUT SURFACE

It has become evident from the data already presented that the cut surface acts on regeneration in some way other than by allowing rapid oxygen passage. The possibility has been considered that regeneration-inhibiting substances produced in the tissues build up to a level incompatible with regeneration unless they can diffuse away. The primary path of exit is through the open stem end. If regeneration is inhibited by some substance or substances released through the open end to sea water, it should be possible to collect the inhibitor in the water.

Small colonies of approximately two hundred stems were cut into pieces, allowed to heal, washed, and put into flasks containing 250 cc. of sea water. Air was bubbled continuously and vigorously through the flasks. After a time water was removed from the flasks and used as a medium for freshly cut stems. At the same time, control stems were placed in untreated sea water. The experiment was performed 17 times with groups of 40 stems. Every time inhibition by colony water was apparent. In general, the amount of inhibition was proportional to the time the water had been in contact with the colony. In Table 5 the data for groups of stems grown in sea water which had been in contact

with a colony for varying times are given. It may be seen that as the time of contact with the colony increased, more stems failed to regenerate, and those which did regenerate did so more slowly.

The data from experiment to experiment is quite variable. Sometimes, for example, 2-hour colony water inhibited but a small percentage of the stems; at other times it almost completely inhibited. However, 12-hour colony water completely inhibited regeneration all four times that it was tested.

Most of the stems remained alive in the inhibiting solution and regenerated when returned to fresh sea water.

The inhibiting action of the colony water must be caused by something released to the water from the tissues, because the water loses its inhibiting power when heated. The inhibitor is thermally unstable. Temperatures of 90°–100° for a few minutes destroy it. The inhibitor is not a gas, as seen from the fact that it was collected in water through which air was bubbling vigorously.

TABLE 5

RELATIONSHIP BETWEEN CONCENTRATION OF INHIBITOR AND DEGREE OF INHIBITION

Hours Inhibitor in Contact with Colony	Number of Stems Tested	Number Completely Inhibited	Percentage Completely Inhibited	Hours' Delay of Stems Regenerating
1.....	40	10	25	5
2.....	40	26	65	8
3·5.....	50	43	86	13
12.....	40	40	100	.....

Cut colonies were not the only source of inhibitor found. Adult hydranths are excellent sources. Ten living hydranths in 50 cc. of sea water with 10 cut stems almost completely inhibited the regeneration of the stems. Regenerating stems themselves inhibit one another. In 16 experiments, the average regeneration rate of 10 stems in 50 cc. of standing sea water was found to be faster than that of 20 or 30 stems in the same volume of standing sea water. The average regeneration time of the 10-stem groups was shorter by from 1 to 8 hours. It appears that both hydranth and stem tissues produce a regeneration-inhibiting substance. Some of the inhibition of stems upon one another in still sea water may be due to substances other than the nonvolatile inhibitor. This can be seen from the fact that the differences in regeneration rates between 10 and 20 stems in bubbling sea water are much less than in still sea water. In 4 experiments the differences between 10- and 20-stem groups in bubbling sea water vary from 0 to 3 hours slower for the larger group. The differences ranged from 1 to 8 hours in the standing water experiment.

It has been shown that substances produced and released by the tissues inhibit regeneration. If the inhibitor is released more readily through a cut surface, free of perisarc, than through an intact surface, then ligatured stems should not inhibit other stems as much as do unligatured stems. An experiment to determine this was conducted in the following way. Stems similar in size and appearance were cut from a single colony.

They were cut to a length of 10–12 mm. Ten were selected at random and placed in a 4-inch finger bowl containing 50 cc. of sea water. Another finger bowl received 20 stems. In a third were placed 10 stems plus 10 ligatured at both ends. The experiment was performed nine times. Each time the average rate of development in the 20-stem dish was slower than that in the 10-stem dish. Furthermore, the average rate of regeneration of the 10 stems housed with 10 ligatured stems was, in all cases but one, intermediate between the regeneration rates of the 10- and 20-stem groups. The data are summarized in Table 6. The one exceptional case, No. 7, may be disregarded, because 4 of the 10 stems did not regenerate at all, and the others regenerated slowly. This is considered evidence that a toxic agent was present in the dish, and therefore this one exceptional

TABLE 6

A COMPARISON OF THE STAGES IN REGENERATION OF STEMS INHIBITED BY LIGATURED AND NONLIGATURED STEMS*

Expt. No.	10 Stems	10 Stems Plus 10 Ligatured Stems	20 Stems
1	50.4†	44.3	42.4
2	35.5†	29.4	21.2
3	21.0†	20.1†	17.2
4	43.7†	40.1†	37.1
5	33.0†	32.2†	29.0
6	46.2	44.3	42.3
7	34.8	17.0	28.7
8	33.9†	29.4	27.9
9	42.0	36.4	35.1

* The morphological stages observed are given the hour-values for the same stages in stems which regenerated at 15° C.

† Indicates average for two groups of stems from same colony done at same time.

case is dropped from consideration. In the 8 experiments, it was found that 10 stems were inhibited by 10 ligatured stems but not as much as they were by 10 stems with open ends. The data seem reliable. The expectation on a purely chance basis for the rate of 10 stems housed with 10 ligatured stems to be less eight times in a row than that for 10 stems housed with 10 open ones is $(\frac{1}{2})^8$.

In the first section of the paper it was demonstrated that stems start to regenerate without an open surface, provided that sufficient oxygen is present, but that they do not continue to regenerate if there is no surface open to sea water. It is now believed that sufficient inhibitor accumulates to prevent regeneration when there is no open surface.

Regeneration-inhibiting substances are produced by the tissues. They are released in sufficient quantity through open ends to permit regeneration. The concentration of the inhibitor must be lower at open ends than elsewhere. Therefore one reason for regeneration close to a cut surface is the relatively low concentration of inhibitor there.

DISCUSSION

The following is a review and discussion of the events leading to the regeneration of a hydranth and the determination of the locus of regeneration.

The first step, and a necessary one, is the isolation of the stem from its hydranth (Child, 1929). Under favorable conditions no extrinsic stimuli are necessary for regeneration. Regeneration commences in isolated stems although the stems are ligatured and the extrinsic factors are unchanged. A cut is unnecessary, as seen from the observation that regeneration can occur in nature during the winter in the stems of intact *Tubularia* without any cut. Possibly the old hydranth must be removed, because, when present, it prevents regeneration by removing from the circulation the raw materials used in hydranth formation. Support for this belief comes from experiments on other Hydrozoa. When a head and upper body of a *Hydra* were grafted onto the body of another *Hydra* (King, 1901), the graft appeared, in the course of days, to move slowly down the body of the host until it reached the base, where it separated from the host. Such apparent motion by the graft through the host tissues might result from continual absorption of more basal regions and faster growth in the more apical regions. The vital staining experiments of Kanajew (1930) indicate that, during the growth of the head in *Hydra*, growth proceeds by the addition of cells which were formerly body cells. Goetsch (1929) gives evidence that, during regeneration, cells from other regions migrate into regenerating regions. These facts show that the head region removes materials from more basal regions during growth and during regeneration. It is thought that an adult hydranth removes these materials rapidly enough to inhibit regions which might regenerate if they had the raw materials.

Several factors work together in determining the locus of regeneration. Distal regions have an inherent advantage over more proximal regions. Other factors promoting regeneration locally are a relatively high local oxygen supply and a relatively low local inhibitor concentration. When both distal and proximal ends are open, both ends have relatively high oxygen supply and relatively low inhibitor concentration. The inherent gradient structure in that case determines the place of regeneration. When a narrow-mouthed cap with oxygen is placed over a distal end and left there, the distal region is favored by its gradient structure and the high oxygen, but inhibiting substances prevent the regeneration there. When a large-mouthed cap with oxygen is placed over a distal end, the inhibitor concentration is slightly greater in the distal region but the high oxygen and gradient determine the regenerant there. If, as in the last case, a large-mouthed cap, but without oxygen, is applied distally, the balance is swung the other way by the difference in oxygen concentration. Thus, all three—gradient structure, inhibitor, and oxygen—are shown to play a part in determining the locus of regeneration.

There may be a common mode of action of all these factors which influence the locus of regeneration. All may alter metabolic rate locally and consequently change the rate of raw material removal from the coelenteron locally. Evidence presented by Child and his associates (see reviews by Child, 1929, and by Barth, 1940*b*) indicates that the region in which a regenerant forms metabolizes more rapidly than do other regions. Distal regions have higher rates of oxygen consumption than proximal regions (Hyman, 1926; Barth, 1940*a*). High local oxygen supply at a cut end would increase the metabolic rate, since oxygen consumption increases with increased oxygen tension (Barth, 1940*c*). Whether the inhibitor also affects the metabolic rate is not yet known. It was suggested

by Miller (1939) that oxygen and high temperature which favor regeneration locally do so by increasing the metabolic rate. An increase in the metabolic rate would presumably be followed by a more rapid extraction from the coelenteron of the raw materials of regeneration.

SUMMARY

1. Removal of dominating regions is the stimulus to regeneration.

2. Regeneration can commence without the stimulus of an open surface.

3. It has been demonstrated that an inhibitor to regeneration is produced by the tissues of *Tubularia*.

4. A cut surface is necessary during regeneration because through it pass oxygen and regeneration-inhibiting substances.

5. The locus of regeneration is determined by the interaction of three factors: the stem level, the oxygen concentration, and the inhibitor concentration.

6. Supporting evidence for the circulation theory of dominance is presented.

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STUDIES ON HOMOPLASTIC AND HETEROPLASTIC TRANSPLANTATION OF METANEPHRIC PRIMORDIA TO THE COELOM OF THE CHICK EMBRYO

(Two Plates)

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THE many diverse experiments in which animal tissues have been transplanted have demonstrated that most adult vertebrate hosts are refractory to heteroplastic tissues from adult vertebrate donors. In contrast, tissues transplanted between early vertebrate embryos have usually lived and differentiated. This dissimilar behavior of embryonic and adult hosts makes it apparent that species specificity has its origin sometime during development.

Several experiments in which transplants have been placed on the chorio-allantoic membrane of the chick embryo have provided evidence of the time of origin of specificity in this form. Among the earliest of these investigations was that of Murphy (1913). He found that embryonic rat tissue grew upon the membrane for periods up to 11 days, but that implants of the same tissue to the just-hatched chick degenerated within 3 days. From these results Murphy concluded that the qualities which make the adult chicken an unsuitable host for heteroplastic tissues develop during the last 2 days of intrashell life. In further experiments Murphy (1914) inoculated the chorio-allantoic membranes of 12- and 15-day chicks with macerated rat sarcoma. The resultant grafts were removed at intervals beginning on the seventeenth day of host incubation. Active growth of the engrafted tissue continued through the eighteenth day but ceased on the nineteenth day, when connective tissue began to penetrate the grafts at their peripheries. Necrotic centers developed by the twentieth day, at which time polymorphonuclear leucocytes and round cells began to enter the grafts. At this stage connective-tissue capsules had formed around the grafts, and strands had begun to penetrate the central regions. The preceding observations were taken as evidence that the specificity of the host appeared suddenly rather than gradually, and the statement was made (p. 521) that "the chick at about the time of hatching develops a defensive mechanism against the tissue of foreign species." Subsequent experiments gave the same results, and Murphy (1926, p. 15) again observed that ". . . . the avian embryo has no defensive mechanism against the growth of heteroplastic tissues till after the eighteenth day of incubation, but after this period it develops rapidly and attains its full power at or about the time of hatching."

The experiments of Murphy have thus established the hatching period as the definitive time during which the chick becomes reactive. A recent statement by Oakley (1938) demonstrated that Murphy's conclusions have found acceptance. On the basis of the results of Murphy (1913), Oakley stated (p. 115) that ". . . . the chick's individuality has developed at hatching to a degree rendering impossible the development of even embryonic tissue of the same species." However, the grafts which provided most of the evidence upon which Murphy's conclusions were based were removed from the chorio-allan-

toic membranes of their hosts in the interval between the seventeenth day of host development and the hatching period. It is a familiar fact that during this interval the blood supply to the membrane diminishes steadily and its value as a functional adjunct decreases accordingly. Since the period during which the chorio-allantoic membrane provides optimum vascular conditions to engrafted tissues ends before hatching, transplants intended to serve as indicators of specificity origin should be established within the body tissues of the host during the definitive period in question. In the present experiments homoplastic and heteroplastic embryonic metanephric tissue was transplanted to the coelomic cavities of embryonic chick hosts and removed at different stages of development up to and through hatching.

The present report offers results which bear chiefly upon the problem of the origin of specificity in the chick embryo and the relation of specificity origin to reactive tendencies in the host. Other factors considered are (*a*) the ability of incorporated and functional tissues to survive the period indicated to be that during which species specificity is assumed to arise in the chick embryo and (*b*) its corollary, the possible relation of the lack of active functioning of transplants to their degeneration.

MATERIAL AND METHODS

White Leghorn chick embryos were used as recipients throughout and served as donors in the homoplastic experiments. Heteroplastic donor tissues were obtained from White Pekin duck embryos.

Transplantation of donor rudiments to the coelomic cavities of chick embryos was first reported by Joy (1934). This investigator placed eye rudiments from 36-hour embryos into the coeloms of 48-hour chick hosts. Later, Hamburger (1938) described a method by which limb primordia were introduced into the coeloms of 48-hour chick hosts through a slit in the somatopleure close to the lateral edges of somites in the region of operation. Eastlick (1939*a*, *b*, and *c*) employed this technique, using 60- and 70-hour hosts. In the experiments reported here, embryos of 72–80 hours' incubation were selected as hosts. In beginning an operation a small square window was made in the shell. This window was lifted to expose the embryo, following which a slit was made in the vitelline membrane and the forming chorion along a line parallel to and just beside the right body wall. The tissue to be implanted was pushed into the coelom through the slit thus made and usually came to lie above the omphalo-mesenteric vessels.

The embryos were operated under a dissecting microscope providing a magnification of 43.2 times, using intense light. Glare from the surface of the embryo was controlled by filtering light from the illuminator through a sheet of Polaroid. Also disks of this material were fitted upon the diaphragms of both oculars of the microscope. Glare was thus eliminated, and the light intensity was modified by rotation of the oculars. Donor tissues were dissected in sterile 0.9 per cent NaCl, on a warm stage. They were freed of the connective tissue by which they were bound to the Wolffian bodies, and all associated tissues were removed. In the homoplastic experiments, $\frac{1}{2}$ metanephros was implanted at each operation. Since the donor tissues in the heteroplastic operations were somewhat older, smaller fractions of the whole gland served as transplants.

The grafts obtained were fixed in Zenker-formol, sectioned at 6 and 8μ and stained in either haematoxylin-eosin, haemalum-eosin, or haematoxylin-azure-eosin. Normal stages of metanephroi were used as bases for comparison with the grafts obtained.

OBSERVATIONS AND RESULTS

Observations have been made on three series each of homoplastic and heteroplastic transplants. The series comprising each group differed by the increasing time intervals between operation and graft removal. The time relations of the hosts, donors, and grafts of these series are set forth in Table 1.

Most of the grafts recovered had become incorporated on or within the mesenteries, since in each operation the donor rudiment was introduced into the coelom at a point near the omphalo-mesenteric vessels. As a result, grafts removed from the mesenteries were most often established near these vessels and received branches from them. A few transplants became attached to the developing liver, mesonephroi, body wall, and mesenteries in the manner described by Joy (1934, 1939). Blood supply and connective-tissue incorporation developed regardless of the locus of implantation.

TABLE 1

TIME RELATIONS OF HOSTS, DONORS, AND GRAFTS

	Group I Homoplastic			Group II Heteroplastic		
Series	1	2	3	4	5	6
Donor age (in days)	7	7	7	10	14	14
Time on host (in days)	5	11	16	5	15	17–18
Age at removal (in days) Host	8	14	19	8	18	20–21
Age at removal (in days) Donor	12	18	23	15	29	31–32
Number of operations	82	73	41	14	22	49
Number grafts recovered	27	16	6	8	7	4
Number grafts studied	27	15	5	8	7	4

THE HOMOPLASTIC GROUP

Tissue incorporation in each homoplastic series was rapid. Well-organized stroma, probably of host derivation, was found in grafts of Series 1, which remained on the host but 5 days. This stroma was especially abundant in areas surrounding renal parenchyma and in the centers of the grafts. Arterioles and venous pathways ramified through the grafts, providing the stroma and renal parenchyma with an adequate blood supply (Pl. I, Fig. 1). These conditions of incorporation were found throughout the six series.

Ser. 1.—The kidney tissue in grafts of this series was equivalent in age to that of a 12-day chick embryo. The development which had occurred gave ample evidence of tissue maintenance and growth. Arborizations of kidney ducts had formed renal trees, and in a few such trees the lumina of collecting ducts were continuous with those of metanephric spheres. An occasional sphere had developed into a capsule with distinct visceral and parietal layers, and, although the majority of these capsules were without vascular supply, a few glomeruli were observed.

Proximal convoluted tubules had developed in small numbers but were not of general occurrence. Their normal differentiation was indicated by the presence of brush borders and striae in the tubule cells. Cytoplasmic vacuoles were disposed in tangential strata in the distal thirds of some of the cells. The cells of the proximal tubules had developed a pink staining reaction which, coupled with the fact that chick kidney tissue 12 days of

age is normally functional (Gersh, 1937), suggested that excretory activity had begun in these tubules.

A stainable precipitate, which apparently resulted from the fixation of a viscous fluid, was found in the lumina of proximal convoluted tubules, collector ducts, and large ureteric branches. This precipitate stained pink in the lumina of the proximal tubules, while a blue reaction was typical in the larger ducts. Occasional phagocytes were present in the lumina of the larger collector ducts.

The ureters of the donor metanephroi were cut through in preparing implants. The ends of the ureters closed after transplantation, and the nephric systems which developed from the rudiments failed to communicate with the coelomic cavities of the hosts.

The stroma of the grafts was loosely organized in the central areas and was present in greater amounts than a supportive function would require. Peripherally, the connective tissue of the grafts was dense, and the grafts were inclosed by a capsule a few cells thick. In well-organized grafts, connective-tissue trabeculae radiated from the graft centers to afford support and convey blood channels to the parenchyma. Malpighian bodies were distributed peripherally in grafts of this type, and the closed ureteric stumps occupied positions corresponding to kidney hila.

In the loose stroma surrounding venous sinusoids, many connective-tissue cells were observed which were in the initial stages of hemoblast formation. In later stages of differentiation, fibrillae had disappeared, and pseudopodia had formed (Pl. II, Fig. 9). The resultant hemoblasts were the apparent source of granuloblasts and granulocytes associated with them in the connective tissue around venous sinusoids (Pl. II, Fig. 10).

Large cells with yellow-appearing cytoplasm were present in almost every graft but occupied no specific position in any graft. Their nuclei were usually basophilic, although some were yellow. While these cells occurred in grafts throughout the series and their appearance was the same in every graft, they did not appear to be renal cells and were never components of organized structures.

Ser. 2.—These grafts, removed from 14-day embryos on the eleventh postoperative day, provided donor tissue 18 days old. Growth of the parenchyma had occurred, but there was no discernible differentiation of either ascending or descending loops of Henle or distal convoluted tubules. Malpighian bodies, normal in form and having glomeruli, had in a few cases developed lumina within which a precipitate was observed. The proximal tubules were longer and of greater diameter than those of the preceding series. The cells of these tubules were acidophilic, possessed brush borders and vacuoles, and displayed cell striation comparable to the normal. Residue found in the lumina also stainde pink. Collector ducts and ureter branches were larger than those of the younger grafts but were unchanged in essential structure. Occasional phagocytes were present in their lumina. The general development of the renal parenchyma of these grafts lagged behind that of normal metanephroi of corresponding age.

The connective-tissue organization in grafts of this series was more complete than that found in grafts of the preceding series. Capsular stroma was heavier, being eight or ten cells thick in representative cases. In the graft centers, loose connective tissue surrounding venous sinusoids contained many hemoblasts as was the case in the younger transplants. However, granulopoiesis was more intense, and the proportion of granulocytes to hemoblasts had increased.

Six of the fifteen grafts examined were necrotic and displayed the following phenomena: (*a*) kidney tubules had lost their characteristic organization and had assumed the

form of solid cylinders; (*b*) no Malpighian bodies were identified; and (*c*) cells of the nephric tissues were vacuolated and deep staining, and some sent ramifying processes to the connective tissue surrounding them. Many hemoblasts, round cells, and connective-tissue wandering cells were present, and a scarcity of supportive cells indicated that connective-tissue cells had been expended toward the production of blood elements.

Ser. 3.—Twenty-three-day metanephric tissue was obtained in these transplants, which were removed from 19-day embryos 16 days following implantation. The renal elements of the grafts presented no striking differentiation beyond the condition of these structures in the transplants just described.

Although most of the proximal convoluted tubules appeared normal, some of them displayed a modified histology. In the latter, cells were observed which contained cytoplasmic vacuoles varying in size from the normal small ones to enormous intracellular spaces which surrounded cytoplasm-enveloped, pyknotic nuclei. In several instances cells apparently had burst and ejected nuclei and cell debris into the lumina of the proximal tubules.

Connective-tissue establishment was different from that heretofore described only in being slightly advanced in organization. Hemopoiesis occurred in positions in which there was much vascular tissue.

THE HETEROPLASTIC GROUP

The connective-tissue organization, vascular supply, and general nature of incorporation of grafts comprising this group corresponded with those of the homoplastic transplants.

Ser. 4.—Implants of duck metanephric tissue from 10-day embryos remained on chick embryo hosts for 5 days. Kidney capsules had developed in the 15-day tissue thus obtained, and some of these were supplied with glomeruli. As in the homotransplants, cells of the proximal tubules were acidophilic, and some contained vacuoles. The remaining nephric elements commonly present in the homoplastic grafts were represented and appeared to have developed in an orderly fashion. Hemopoiesis occurred in the same positions as in the homotransplants. Hemoblasts were more numerous than granuloblasts and granulocytes.

Ser. 5.—These grafts, formed of 29-day-old duck metanephros were removed on the fifteenth day after operation. The recovered transplants presented evidence of a greatly modified renal structure. In many cases the closed ureters were expanded and appeared as extensive vesicles. This dilatation (Pl. I, Fig. 2) frequently extended through the metanephric tree to the cavities of the Malpighian bodies. Correlative with this general distention, a degeneration of some of the proximal tubules had occurred (Pl. I, Fig. 3). This degeneration, characterized by the vacuolation and rupture of cells, was identical in nature with that observed in the oldest grafts of the homoplastic group.

Notwithstanding the generally abnormal nature of the renal parenchyma, a large number of normal Malpighian bodies and collecting tubules were present. Normal proximal tubules varied among themselves in staining intensity but in no other particular. The commonplace nature of cells of these tubules was indicated by the presence in them of brush borders, striae, and vacuoles which were occasionally disposed in strata.

Hemopoiesis was intense, and granulocytes were the most numerous of the types of blood cells observed.

Ser. 6.—Duck metanephros from donors of 14 days' incubation was allowed to remain engrafted until fixation just after the hatching of the hosts. In spite of the advanced age of the graft tissue, renal structures, when normal, were entirely representative (Pl. I, Fig. 4; Pl. II, Fig. 8). Many of the Malpighian bodies were normal and vascular, but a large number of capsules contained cavities within which detritus and nuclei were found. The majority of proximal tubules were abnormal and displayed ruptured cells and lumina which contained nuclei and debris (Pl. I, Fig. 5; Pl. II, Fig. 7). The proximal ends of some tubule cells remained adherent to their basement membranes, while the remaining portions of the cells were discharged into the duct cavities. Some of the tubules were represented by a basement membrane invested by a thin layer of connective tissue. The persistence of a space bounded only by basement membrane and its investing stroma and of greater diameter than the normal proximal tubule indicated that fluid pressure maintained the turgor of these degenerate tubules. Nuclei and cell parts, associated with a precipitated substance, were present in the lumina of some of the degenerate proximal tubules. The precipitate was in small, stellate clumps, from which thin processes extended to the basement membranes of the tubules. A few leucocytes were associated with these clumps, but there was no apparent reason for their presence.

Lumina of the smaller collecting ducts sometimes contained free nuclei and cell fragments. The large ureteric branches were distended, and it is probable that fluid contained within these branches caused an observed flattening of the normal columnar epithelium. The ureter and its large tributaries were incased in a thick tunic of connective tissue, indicating response to a necessity for support created by internal pressure.

GENERAL CONSIDERATIONS

Examination of the grafts comprising all the six series has brought to attention certain features which were observed throughout the results. One of these features was the regular growth exhibited by the transplanted metanephric rudiments. In grafts of corresponding ages kidney development was identical with that reported by Atterbury (1923) for homoplastic transplants to the chorio-allantoic membrane of the chick embryo. In the older grafts of her experiments Atterbury observed that the ascending and descending loops of Henle failed to develop completely. Such a failure was characteristic of the grafts herein reported.

Another phenomenon of general occurrence was hemopoiesis. The cells of the loose connective tissue surrounding vascular channels gave origin to hemoblasts which were observed in the stages of their transformation (Pl. II, Fig. 9). The further differentiation of these hemoblasts was observed to vary with environmental conditions. If by amoeboid activity these cells traversed the walls of sinusoids to enter the blood stream, further differentiation was toward the production of erythrocytes. The ones which remained outside the lumina of blood vessels gave rise to granuloblasts and granulocytes, which remained in the vicinity of their formation to form large aggregations (Pl. II, Fig. 6). Much of the connective tissue present in the earliest grafts must have been supplied by the hosts, judging from the volume in which it was present. It can thus be assumed that host cells have reacted in accordance with their relation to the vascular channels of the grafts. This environmental control of hemoblast differentiation corresponds to the description of chick hemopoiesis given by Danchakoff (1916) and Atterbury (1923) and is presented here as further evidence of the potentialities of embryonic connective-tissue cells.

The origin of the precipitate observed in all segments of the nephric systems is undetermined. Judging from its volume, it could not have resulted from developmental processes. Since the volume increased with the ages of the grafts, it would be reasonable to assume that the precipitate represents a kidney secretion coagulated at fixation. Although strongly indicated, this origin can only be assumed on the basis of present evidence.

Further indirect evidence of the active functioning of engrafted renal tissues is supplied by the breakdown of cells of the proximal tubules. Since the ureters of the engrafted rudiments closed shortly after implantation, the introduction of a fluid into the nephric lumina apparently resulted in general distention of the kidney system. Such distention was observed in grafts of the older series, and, in Series 3, 5, and 6, proximal tubules were observed which contained vacuolated and ruptured cells. The observations indicate that small normal cell vacuoles, prevented from discharge into the proximal tubule lumina by internal pressure, coalesced to form large vacuoles. Appearances indicate that these, in turn, fused to form a vacuole surrounding the nuclei and their immediate cytoplasm. Further expansion apparently caused cell rupture and the discharge of the cell nuclei into the lumina of the tubules. Because the rupture of these cells occurred largely at the apical ends, the basal membranes of the proximal tubules, along with their investing sheaths, appeared to prevent rupture at the proximal ends of the cells. Secretion against an internal pressure could plausibly have resulted in the degeneration of proximal tubules. The active functioning of the kidney elements which developed in the grafts has therefore been strongly indicated by (*a*) the nature and staining reactions of the precipitate contained within the lumina of the proximal convoluted tubules and (*b*) the dilatation of the nephric systems, accompanied by cytological changes in cells of the proximal tubules which were apparently related to this distention in a direct way.

The peculiar yellow cells gave but one indication of their origin. In a graft of Series 4, a group of metanephric-sphere cells was observed which had neither a cavity nor continuity with the collector system. Although some of these cells had fibrillae and their nuclei and nucleoli closely resembled those of connective-tissue cells, they were much larger than the ordinary supporting cells. These facts suggested that after failure of a metanephric sphere to become established as a kidney unit its cells began to degenerate and appeared in the grafts as moribund elements (Pl. II, Fig. 6).

DISCUSSION

The most significant result of these experiments has been that the hosts did not react in any discernible way against either the homoplastic or the heteroplastic grafts. Connective tissue and blood elements, relied upon as criteria of host antagonism, gave no indication that reaction against the transplants had occurred. It is important in this connection that the aggregates of granulocytes observed arose from stem cells within the grafts themselves. Furthermore, those kidney structures which were present in grafts of all the series had grown without inhibition by the hosts. While there were degenerative changes in some proximal tubules, these found reasonable explanation when viewed as results of the functioning of the secretory elements of the grafts. In short, no degenerative changes were observed which could definitely be ascribed to host antagonism.

It will be recalled that Murphy (1913, 1914, 1926), on the basis of results obtained from heteroplastic transplantations, concluded that the expression of species specificity

by the chick embryo began just before hatching and increased to a potency comparable with that of the adult during the hatching period. The heteroplastic transplants observed in the present experiments failed to give any evidence of such an abrupt and unqualified assumption of species specificity.

In the absence of the clear evidence which host reaction to heteroplastic tissues would have provided, it is necessary to consider whether the implanted tissues became adapted to their hosts or whether reactive tendencies had not developed in the hosts during the period in question. There is much to suggest that the former interpretation is the more acceptable. However, if adaptation of tissues to their hosts is assumed to allow graft persistence, the correlative assumption is that the hosts were capable of reacting. The results of Sandstrom (1934) indicate that this is the case. He found that embryonic duck metanephros from donors of ascending ages was reacted against by the chorio-allantoic membranes of chick hosts shortly after implantation, and to degrees which varied directly with the ages of the implanted tissues. On the basis of this evidence it can be assumed that heteroplastic tissues in the present experiments persisted through hatching because they became adapted to their hosts.

This conjectured adaptability may be explained by factors other than the specificity relations of hosts and donor tissues. For example, the results of many experiments have led to the suggestion that transplants of glandular tissues can survive if they continue their functions in the engrafted condition. The investigations of Kiyono and Sueyasu (1917) are pertinent in this regard. They transplanted chick, duck, turkey, rat, mouse, guinea pig, and rabbit tissues to the chorio-allantoic membranes of chick and duck hosts. The connective tissue, cartilage, and skin components of the grafts persisted longer than did glandular tissues. In this same connection Murphy (1926), studying grafts resulting from the inoculation of hashed chick embryos to the chorio-allantoic membrane, observed that the epithelial elements of the grafts diminished but that bone, cartilage, and connective tissue survived in the transplants. Sandstrom and Kauer (1933) advanced further evidence of the ability of cartilage to survive engrafting. They transplanted cartilage from 20-day duck embryos to the chorio-allantoic membrane of the chick, and, although the resultant grafts were maintained for 27 days by serial transplantation, they presented no evidence of host antagonism. In contrast with the success of passive sustentative tissues as transplants, C. J. Sandstrom (1934) advanced inability to continue functioning to explain the degeneration which he observed in engrafted duck metanephros. Similarly, R. H. Sandstrom (1934) ascribed the eventual failure of pancreatic and liver rudiments on the chorio-allantoic membrane to the same cause. The foregoing evidence indicates that active functioning by engrafted tissues prolongs their survival as transplants.

It has been previously pointed out that the oldest heteroplastic grafts in the present experiments were, judging from all appearances, functional when removed. The question now evolves whether adaptation of a donor tissue to a host, which would explain its survival, consists in the mere establishment and maintenance of such tissue in an environment which allows function. In the present case, assuming that either the donor tissue or the hosts or both were species specific at the time of graft removal, the results obtained present indirect evidence that functional transplants can override those factors which would, in the absence of complete incorporation and tissue function, act to remove them. The nature of the modifications involved and their effects upon hosts and implanted tissues remain obscure.

The alternative explanation of the observed absence of host reactivity rests in the possibility that the host either does not develop a species specificity during the period examined or this specificity is modified by factors not generally encountered in transplantation experiments. In the former event solution of the problem awaits the ascertaining of the actual period during which reactive capacities arise., In the latter case the work of Sandstrom (1936) may offer an explanation. Sandstrom made reciprocal chorio-allantoic transplantations of chick and duck tissues to duck and chick embryos. The duck tissues evoked but mild reactions from the chick membrane, while the chick grafts called forth pronounced reactions. It was concluded that no reciprocity of reactive tendencies exists between the forms examined. On this basis, and considering the present results, the chick may be accepted as an unreactive host to duck tissues. However, such an explanation of the lack of host reaction observed would be based on a host-donor relationship which may not be general among the higher vertebrates. Therefore, the nature of the results of transplantations of kidney tissue from embryos of other vertebrates, perhaps more distantly related, cannot be inferred from the evidence presented here.

It was previously indicated that six of the sixty-six grafts examined were necrotic. It is not believed that this necrosis was due to host reaction, since grafts of identical host-donor relations, allowed to remain engrafted for longer periods, failed to be attacked. In the absence of the specificity factor the results of Rous (1910) provide a possible explanation. He implanted mouse tumor and embryonic mouse tissues homoplastically beneath the skin of adult recipients and found that some of the hosts were more resistant to embryonic mouse tissue than others. In the present case the hosts from which the necrotic tissue was removed were embryonic, as was the tissue of the transplants. It is hardly likely, therefore, that either host or donor tissue was reactive and improbable that the observed graft failure resulted from antagonism by atypically reactive individuals.

On the other hand, simpler factors may explain the degeneration observed. It is possible that the blood supply was adequate for maintenance and growth of the grafts during the first few days of their residence upon the hosts but that vascularity did not increase with the subsequent growth of the tissue. Superior relative growth of the kidney tissue may thus have resulted in the necrosis observed. Finally, the necrosis may have been due to tissue injury during transplantation. In this case the degeneration would not be an index of either the suitability of the site of transplantation or the conditions required for the survival of the tissue engrafted.

SUMMARY

1. Six series of transplantations of chick and duck metanephric rudiments were made to the coeloms of 72–80-hour chick hosts. The resulting grafts were allowed to remain in the host for periods varying from 5 days to 17 and 18 days and were removed from hosts which varied in age from 8 days to just-hatched chicks.

2. Four heteroplastic grafts which remained on host embryos through the period of hatching gave no evidence of having been reacted against. It was therefore concluded that the species specificity of the chick did not appear under the conditions of the experiment.

3. Explanations advanced for this result were based on (*a*) a possible adaptation of donor to host tissues; (*b*) a possible effective resistance of established and functional transplants to host reaction during the hatching period, (*c*) a lack of reactivity of chick

hosts to duck donor tissue, and (*d*) an absence of species specificity in the chick embryo during the period covered in the experiment.

4. The transplants presented histological evidence of (*a*) active functioning by the kidney elements of the grafts and (*b*) hemopoiesis similar to that described by Danchakoff (1916).

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PLATE I

FIG. 1.—×100. Section of graft of Series 1 to demonstrate conditions of incorporation which prevailed throughout the entire experiment. Observe: (1) relation of graft to gut (*a*), mesentery (*b*), and blood supply (*c*); (2) organized stroma; (3) typical distribution of vascular channels and loose connective tissue within the graft; and (4) well-established renal parenchyma.

FIG. 2.—×100. Section of graft from Series 5 which illustrates the extensive dilatation of the entire collector system.

FIG. 3.—×400. Section represented in Figure 2 more highly magnified. Observe the vacuolation of cells of the proximal convoluted tubules (*a*), the distention of these tubules, and the debris in the duct lumina (*b*).

FIG. 4.—×100. Graft of Series 6. Presents the generally normal appearance of those transplants allowed to remain on their hosts through the hatching period. Compare the general character of the transplant with that of Figure 2.

FIG. 5.—×400. Graft of Series 6. Gross appearance of the parenchyma of a graft which remained on the host through hatching. Precipitate and cell debris may be observed in the duct lumina. Compare with Figure 3.

PLATE II

FIG. 6.—×400. Graft of Series 5. The cluster observable in the upper center (*a*) is composed of yellow cells. The cells aggregated in the upper right (*b*) are granuloblasts and granulocytes. These elements are seen to occur in areas bounding renal parenchyma (*c*).

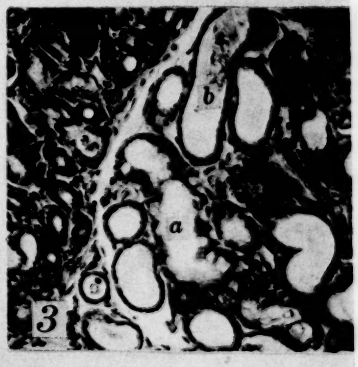
FIG. 7.—×950. Proximal convoluted tubules undergoing degeneration. From the graft represented in Figure 5.

FIG. 8.—×950. Normal proximal convoluted tubule (*a*), containing brush border. In the same field are tubules in the process of degeneration (*b*). From the graft represented in Figure 5.

FIG. 9.—×950. Amoeboid hemoblasts in the loose connective tissue of a graft of Series 4 (*a*).

FIG. 10.—×950. Granuloblasts (*a*) arising in loose stroma surrounding venous sinusoid (*b*).

PLATE I



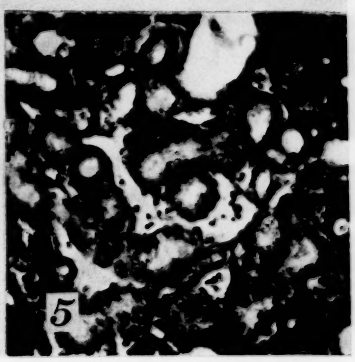


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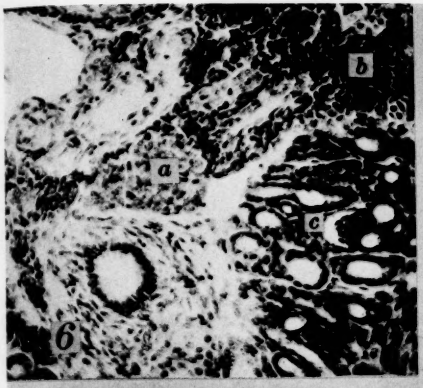
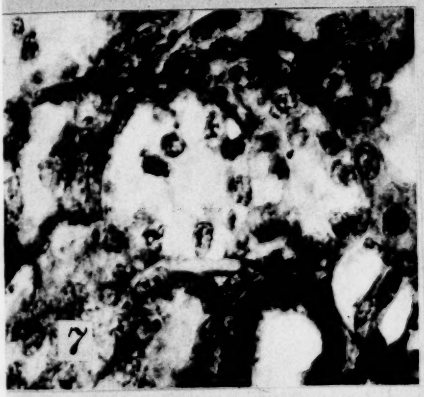
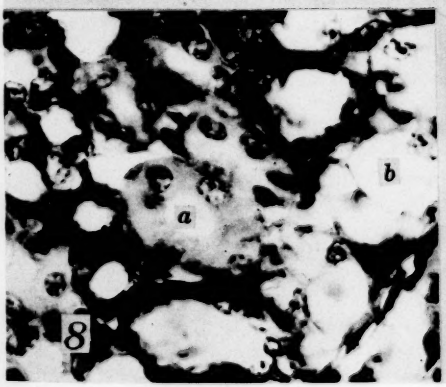
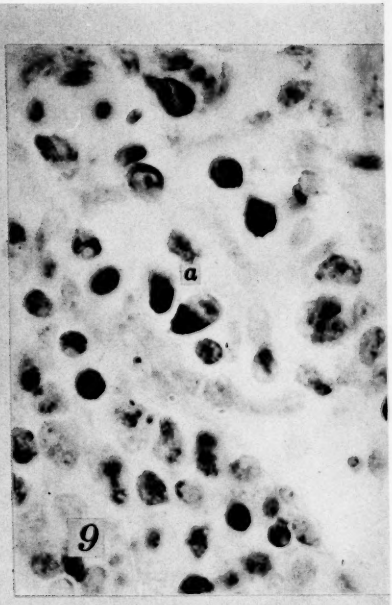
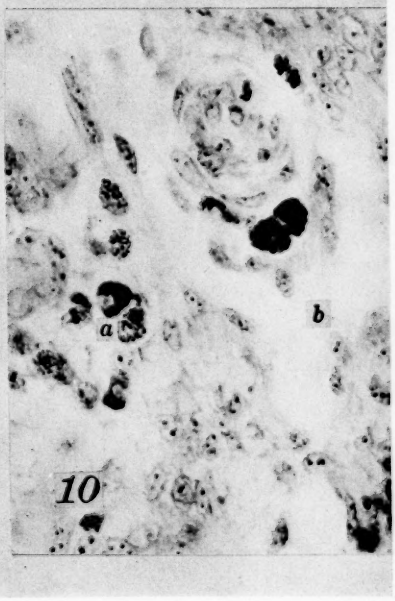
PLATE II











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TRANSPLANTATION OF LIMB PRIMORDIA OF HOMOZYGOUS AND HETEROZYGOUS CHONDRODYSTROPHIC ("CREEPER") CHICK EMBRYOS[1]

(One plate)

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IN THE experiments to be presented in this paper an attempt has been made to analyze certain aspects of the action of the "Creeper" factor in the chick, using the method of embryonic transplantation. The "Creeper" material has been chosen because the effects of this hereditary factor on development have been studied to a considerable extent by Landauer and his associates (1931–40); thus a sound basis for further investigations has been laid.

Landauer and Dunn (1930) have shown that this mutation is a dominant lethal and that the heterozygous phenotype is identical with chondrodystrophy described for mammals. In the heterozygous condition (Cpcp) the "Creeper" factor is expressed mainly by a considerable shortening of the legs. The tibia is bent, the fibula is abnormally strong and nearly of the full length of the tibia, with which it is fused on both ends. In the homozygous condition (CpCp) the "Creeper" factor is usually lethal at the fourth day of incubation. At that stage the embryos are retarded considerably in growth and differentiation; the heads are disproportionately small and asymmetrical, blood pools are apparent both intra- and extra-embryonically. A small number of homozygotes survive this critical period, but they never reach the hatching stage. They are characterized by a considerable growth reduction. Wings and legs are extremely short, the latter being rotated craniad. The feet seem to be attached directly to the flank, a condition which is known as "phocomelia" and occurs as a rare congenital malformation in mammals and man. The skull and eyes show marked abnormalities. Throughout the present paper these two types of homozygous embryos will be designated as "early lethal" and "phocomelic" embryos respectively.

When early primordia of Cpcp and CpCp embryos are transplanted heterotopically onto normal embryos, their genetic milieu is altered as well as their relation to inductive and other correlative factors. Nutrition and innervation will be supplied by the host. A priori, the transplants may be expected to follow either one of two courses. They may differentiate into typical Creeper- or phoco-like structures. This would mean that the gene-controlled processes which result in the phenotypic malformations are, from the moment of operation, entirely intrinsic in the primordium and not modifiable under the conditions of the experiment. In this instance, variations of the stage of operation, of the site of transplantation, and of the genetic constitution of the host would be required for further analysis. The other alternative would be that the transplants would not exhibit

[1] This investigation was aided by a grant from the Rockefeller Foundation. I wish to express my appreciation of the valuable assistance of Mr. Eugene L. Keefe. The results were briefly reported in Hamburger (1939*b*).

the characteristic Creeper or phocomelia features; they might even be expected to develop normally in the normal milieu. The obvious conclusion would be that the primary gene action is extrinsic to the transplant and that the phenotypic expression is brought about by mediation of inductors, hormones, or other agents. Different primordia, and even partial developmental phases of the same primordia, might behave differently in this respect.

Of particular interest will be the fate of transplants taken from "early lethal" embryos. The fact that some embryos of CpCp constitution do survive and the results of tissue culture and chorio-allantoic grafting experiments by David (1936) indicate that lethal tissue is viable beyond the critical stage (the fourth day of incubation). The transplantation of such primordia into an optimal milieu should reveal their full morphogenetic and histogenetic potencies and supply additional information concerning the causes of lethality of the CpCp condition.

With these possibilities in mind we chose limb primordia as the first test objects because they are affected most drastically by the Cpcp and by the CpCp genotype and because the method of the limb-bud transplantation had been worked out previously. Fortunately, the early lethal embryos are still alive at a stage when their wing and leg primordia are easily accessible to transplantation.

MATERIAL AND METHODS

Material.—The Creeper material came from the same stock of the Storrs Agricultural Experiment Station, Storrs, Connecticut, as that which had been used by Landauer and his co-workers.[2] The material used for hosts was a pure-bred strain of White Leghorn raised in St. Louis County, Missouri.

Method of operation.—The method of limb-bud transplantation has been described previously (Hamburger, 1938, 1939*a*). Since the Cpcp embryos cannot be distinguished from normal embryos at the time of operation (third day of incubation), an attempt was made to raise the donor embryos. This necessitated a slight modification of the operative procedure. A window was made in the shell of the donor embryo. Its leg-forming area was vital stained *in situ* with neutral red, cut out by means of a glass needle, and transferred in a micropipette to the previously prepared host embryo. The windows of donor and host embryos were sealed in the usual manner and both embryos returned to the incubator.

Stages at operation.—The donor embryos ranged from 49 to 74 hours of incubation (19 to over 30 somites). The limb primordia varied between Stages 1 and 5 (Hamburger, 1939*a*, p. 349), i.e., from stages in which the thickening of the limb area was not yet visible externally to those of medium-sized buds. The limb primordia of CpCp embryos (of the same age) are usually retarded and their mesoderm less dense, so that they are less prominent than normal (Landauer, 1932). They are never larger than Stage 3 (slight swellings). The host embryos were between 64 and 72 hours of incubation (limb-bud Stages 2–4).

Site of transplantation.—The limb primordia were transplanted either to the right flank of the host embryo between the wing and leg bud or into the coelomic cavity. Both locations have been shown to be favorable for a normal differentiation of limb transplants. In general, the morphogenesis and the histological differentiation of the limb

[2] I wish to express my deep appreciation of the co-operation of Dr. W. Landauer, of Storrs, who supplied the material and made his collection of slides available for the present investigation.

skeletons proceed normally, except for the joint formation in cases which receive inadequate nerve supply or none at all. The occurrence in the present material of ankylosis and of other deficiencies at the articulating surfaces, characteristic of noninnervated skeletons, are therefore a result of the transplantation as such and cannot be attributed to the Creeper factor (see Hamburger and Waugh, 1940).

DIAGNOSIS OF THE DONOR EMBRYOS

CpCp embryos.—Early lethal embryos can usually be recognized at the beginning of the third day of incubation (Landauer, 1932; Rudnick and Hamburger, 1940). The most reliable criteria are shortness and incomplete flexure of the head; asymmetries in the head, left eye and ear primordia being smaller than right ones; blood pools occurring intra-embryonically and in the area vasculosa, particularly at the level of the leg primordia. Most of the donor embryos, selected according to these criteria, were fixed after operation and the diagnosis reaffirmed on whole mounts. Of 45 embryos diagnosed as early lethals, 23 gave good transplants, and all showed unmistakable CpCp characteristics (see Table 1). To these, 1 transplant was added which showed the same typical

TABLE 1

SURVEY OF LIMB TRANSPLANTATIONS[3]

Donors	Total	Host Died	Transplant Absent or Poorly Developed	Transplant Well Developed	Genetic Constitution of Positive Cases		
					Cpcp	cpcp	Doubtful
CpCp.....	46	14=31 per cent	8=17 per cent	24*=52 per cent	.....	.....	.....
Cpcp and cpcp..	56	14=25 per cent	9=16 per cent	33 =59 per cent	19†	10‡	4

* Seven wings, 17 legs. † Seven donors raised. ‡ Two donors raised

CpCp features but whose donor, at 71 hours of incubation, had been protocoled as "probably normal though anemic and slightly retarded." This embryo was, in all probability, a phoco-embryo which at that early state was not identifiable as such.

Identification of Cpcp and of normal (cpcp) embryos.—These two classes are indistinguishable on the third day of incubation. The direct way of establishing their genetic constitution by raising the donor embryo to an age of 11 days or older was successful in 9 cases; 7 of these were identified as Cpcp and 2 as cpcp. The transplants from the latter were normal, as expected; all 7 transplants from the Cpcp donors showed typical Creeper-like abnormalities (see Table 1). This difference between Cpcp and cpcp transplants provided a basis for the identification of those transplants whose donors had died too early. Twenty such transplants segregated clearly into two groups: 12 with Creeper-like malformations and 8 transplants with no other deficiencies than are characteristic for normal transplants (ankylosis, occasional hypodactyly, etc.). Four doubtful cases were discarded. If we consider the 12 Creeper-like transplants as of Cpcp constitution, then we obtain a ratio of 19 Cpcp: 10 cpcp.[3] The fact that this ratio is almost exactly the expected ratio 2:1 is considered further evidence that our diagnosis is correct.

Table 1 summarizes the results.

[3] These figures differ from those given in a preliminary report (1939*b*). A careful re-examination of the material resulted in the elimination of several cases; a few new cases have been added.

DESCRIPTION OF THE Cpcp TRANSPLANTS

Only leg primordia were transplanted, since the wings are not markedly affected by the Cp factor. Most embryos were fixed at 11–12 days of incubation, a few at 16–17 days of incubation. Eighteen specimens were stained in methylene blue, and 1 was sectioned. For comparison the following were available: (1) Creeper embryos (donors and nonoperated specimens of corresponding stages); (2) normal embryos (hosts and nonoperated embryos); (3) a large material of leg transplants of normal onto normal embryos.

Nonoperated Cpcp legs, at 11–12 days of incubation (Fig. 1), show the following characteristics (Landauer, 1927 and 1931): All elements are considerably shorter than normal. In the more extreme variants the individual elements are more plump and stubby than normal. The femur is usually slightly bent, and, if so, hypertrophied periosteal bone is deposited in the concavity. The tibia and fibula are more severely affected than any other element. The tibia is greatly reduced in size, its diaphysis is bent in a varying degree; the concavity is completely filled with hypertrophied bone. The fibula which in normal legs is a slender rod less than one-half the length of the tibia, is stronger than normal and of the same length as the tibia. In normal legs it articulates with the proximal epiphysis of the tibia and fades out into a fibrous strand at its distal end; in Creeper legs both ends are fused with the two epiphyses of the tibia.

The four best-developed Cpcp transplants (among them two cases whose genetic constitution had been established directly by raising the donor) show exactly the same clear-cut symptoms. Case cl 84 (Figs. 1–3) may serve as an illustration. The transplant (right leg bud, Stage 4, 67 hours of incubation) had developed on the right flank of the host. Both donor and host were fixed at 11 days of incubation. The transplant (Fig. 2) is almost exactly of the same size as the left donor leg (Fig. 1), and both are approximately 25 per cent shorter than the host leg (Fig. 3). Both the donor and the transplanted leg show the characteristic curvature of the tibia, the periosteal hypertrophy at the concavity, and the abnormally long fibula which is fused with the epiphyses of the tibia. Even minor details such as the degree of cartilage erosion in the diaphyses of the long elements are identical in transplant and donor leg and slightly advanced in comparison with the host leg. (This precocity in cartilage resorption is not a constant feature of Cpcp embryos.) In another respect the transplant is intermediate: the individual elements of the donor leg show the characteristic stubbiness of the Creeper type; the transplant cartilages are more slender (cf., e.g., the phalanges). Other transplants are of the Creeper type, however, so that no generalization can be made from this observation.

All transplants except the 4 mentioned thus far show higher degrees of malformation. Two different types of deficiencies can be distinguished. Certain abnormalities represent clearly an accentuated Creeper condition, whereas others are identical with those found in transplanted normal limbs and must be considered as nonspecific effects of the transplantation per se. Both the exaggerated Creeper effect and the transplantation effect may be found in the same specimen. The following are classed as aggravations of the Creeper condition: (1) tibia and fibula fused entirely and represented by a broad, flat cartilage plate; (2) tibia and fibula separate, but shapeless and with no condyles (in one extreme case, they are missing altogether, the femur and three typical toes being present); (3) deformations of the femur, which were found in 4 cases; (4) metatarsals occasionally bent and in rare instances partly fused, as is characteristic of higher degrees

of nonoperated Creeper legs. All these features, in particular the malformations of tibia and fibula, are exaggerations of what is found in Creeper legs and are in striking contrast to the abnormalities found in normal, transplanted legs. In an unselected material consisting of 40 normal leg transplants, an abnormally short tibia was found only twice. In 1 of these cases it was bent in a Creeper-like fashion. The fibula of normal transplants was frequently missing (9 cases) but never showed a tendency toward growth beyond its typical proportions. Superimposed on these Creeper effects are others which are characteristic of transplanted normal legs (Hamburger and Waugh, 1940) but which never occur in nonoperated Cpcp embryos. Most frequent among them are hypodactyly and hypophalangy and a combination of both. Hypodactyly was found in 11 of 19 Cpcp transplants, the smallest (first) toe being most frequently absent, as is the case in normal transplants. Ankylosis is common in noninnervated limbs and is definitely not a Creeper effect but due to lack of innervation.

Growth reduction.—Landauer (1934) has made an extensive study of the growth reduction of the different elements of the Cpcp legs. He found differential effects, the tibia being most excessively reduced in length. In general, he found that the growth reduction is in proportion to the final length of each element and that a proximo-distal gradient of growth retardation exists.

In order to obtain an approximate estimate of the size reduction of the Cpcp transplants, the lengths of their femora were measured and compared with the femur lengths of donors and hosts wherever they were available or with the data given by Landauer for the femora of Creeper and normal legs of corresponding stages (Landauer, 1934, Tables 3 and 4). The tibia was not chosen on account of its atypical shape. In the stages under consideration the average length of a nonoperated Cpcp femur is 20 per cent below that of a nonoperated cpcp femur. The transplanted Cpcp femora were found to be approximately 40 per cent shorter than nonoperated cpcp femora (average of 10 cases) which is a growth reduction of 20 per cent in excess of the Cpcp donors. This additional growth restriction can be accounted for readily since we have found previously that normal leg transplants rarely reach the full size of normal legs. The average growth reduction of the femur was calculated as 20 per cent (Hamburger and Waugh, 1940, Table 2). This is exactly the amount by which the nonoperated Cpcp femur exceeds the transplanted Cpcp femur. The total growth reduction of the Cpcp transplants (which is proportional to that of the femur) can be considered as the cumulative effect of the Cp factor and the transplantation per se, each contributing 20 per cent. It follows that the Cpcp transplants have retained their intrinsic limited growth rates as well as their morphogenetic characteristics.

DESCRIPTION OF THE CpCp TRANSPLANTS

As has been indicated previously, all donors except one (probably a phoco-embryo) were identified as early lethal embryos at the stage of operation. Both wing and leg primordia were transplanted. Most of the successful cases (17 legs, 7 wings) were fixed at 12 days of incubation; a few were incubated for 17 days. Four specimens were sectioned; the others were stained in methylene blue.

Table 1 shows that limb primordia taken from early lethal embryos shortly before death not only survive but yield almost as high a percentage of well-developed transplants as that of the Cpcp series. All CpCp transplants were found in a healthy condition. The viability of limb primordia is not impaired by the CpCp genotype.

The appearance of all transplants of this group is very uniform: they are extremely small and resemble closely the phocomelic limbs of those CpCp embryos which survive the critical fourth day of incubation ("phoco-embryos"). The skeleton of the latter was described by Landauer (1933, 1939) as follows: The legs are rotated 180° in their longitudinal axis and point cephalad. Their proximal elements are extremely shortened so that the foot seems to be directly attached to the body wall ("phocomelia"). The fibula is almost as large as the tibia and thus resembles the Cpcp condition. Both tibia and fibula are fused in a varying degree. The same holds for radius and ulna. In some specimens femur and humerus are present only as extremely short rudiments. Hypodactyly was found occasionally in legs. Striking abnormalities were discovered in the histological structure of the skeletal elements: calcification and periosteal ossification are completely inhibited, and the cartilage zones characteristic of the epiphyses of the long elements are absent. Our own material of nonoperated phoco-embryos stained in methylene blue showed, in addition, that the long elements are frequently flattened and atypical in shape. Their articulating surfaces are not clearly sculptured, condyles, etc. are barely indicated, yet the articular clefts are present. The abnormalities in metacarpals, metatarsals, and phalanges are even more pronounced (Figs. 4 and 6). Usually all elements of a digit or a toe are fused into a long, tapering cartilaginous rod with no indication of the typical hourglass shape of the phalanges. The joints are either completely missing or indicated by a fine slit. The boundary between metatarsals (or metacarpals) and first phalanges is usually indicated by an abrupt narrowing of the rod. The proportions of digits and toes are rather typical despite the fusions.

The condition of the best-developed CpCp transplants differs surprisingly little from this description (cf. Figs. 4 and 5; Figs. 6 and 7). The transplants show frequently an additional abnormal feature—the fusion of adjacent long elements. Such ankyloses are characteristic of nerveless transplants, as was mentioned above. However, it is doubtful whether, in the present case, the fusions can be attributed entirely to the lack of innervation, since the condition in the distal parts of nonoperated phocomelic limbs indicate that the Cp factor in a double dose has the tendency to affect skeletogenesis in the same direction. The abnormalities in the shape of the individual elements, together with the fusions and the complete absence of typical articulating surfaces, tends to eliminate the characteristics of the individual cartilages. Only their relative positions and size relations are maintained.

A number of specimens represent higher degrees of malformation, as was the case in the Cpcp transplants. Tibia and fibula were occasionally fused completely; radius and ulna remained more frequently separate, though they might be fused at their ends. Hypodactyly (sporadic in nonoperated phoco-legs and in normal leg transplants) was again quite common in legs, though less frequent than in Cpcp transplants, and usually resulted in the loss of the first (smallest) toe. All but one wing showed the complete number of digits. Again, these deficiencies must be attributed, in part, to the transplantation per se.

The 4 sectioned cases (12 and 17 days of incubation) showed all the histological characteristics found by Landauer in phoco-limbs of the same stages: absence of typical cartilage zones at the epiphyseal ends, complete absence of periosteal ossification, and only the beginning of excavation of the diaphyses. These deficiencies are the more remarkable as the older transplants are incorporated in a host in which ossification and cartilage breakdown are in advanced stages. They demonstrate the complete inde-

pendence of the transplant from the host, with respect to morphogenesis and histogenesis.

Growth reduction.—Recently, Landauer (1939) has published the growth curves of the skeletal elements of phoco-embryos. These data enable us to compare the growth of early lethal transplants with that of nonoperated phoco-limbs which they resemble so closely in morphological respect. Measurements were made on 10 leg transplants, all fixed at 12 days of incubation. The measurements are necessarily crude and give only a rough estimate. The average length of the transplant femur was found to be 2.9 mm., as compared to 3.3 mm. of phoco-embryos of the same stages (=15 per cent reduction). The corresponding figures for the tibia are 2.3 and 3.3 mm. (=30 per cent reduction). This growth reduction in excess of the phoco-legs must, again, be attributed to the transplantation as such. Although only one stage was checked, it appears that the inherent growth rate of a limb bud from an early lethal CpCp embryo is of the same order of magnitude as that of a surviving CpCp leg. Another fact stands out: The tibia is more seriously affected than the femur. This is in full agreement with earlier observations on normal limb transplants, on nonoperated and transplanted Cpcp legs and on phoco-legs; all of which indicate a greater susceptibility of the tibia primordium as compared to the femur.

In summary, wing and leg transplants from early lethal CpCp embryos are viable but show the same restrictions in their morphogenetic, histogenetic, and growth potencies as were found in the surviving CpCp embryos.

DISCUSSION

VIABILITY OF PRIMORDIA FROM EARLY LETHAL EMBRYOS

The survival of the phocomelic embryos proves that CpCp structures are viable under certain conditions. David (1936) has shown the same for several tissues of early lethal embryos. He reared isolated heart, limb, and other primordia of 72-hour CpCp embryos *in vitro* and as chorio-allantoic grafts. In tissue culture they remained healthy and retained their growth activity for as long as 27 days. Chorio-allantoic grafts included well-differentiated, though not typically shaped, cartilages from limb primordia; furthermore, muscle and nervous tissue and feather follicles were present. Histological differentiation was normal except for the limb cartilages, in which the cartilage zones were absent. The same deficiency is characteristic of nonoperated phocomelic limbs and was found occasionally in chorio-allantoic grafts of limb cartilages from normal embryos. Our own material demonstrates a high degree of morphogenetic potency in addition to growth and histogenesis. Healthy legs and wings of the phocomelic type, including cartilaginous skeleton, musculature, tendons, and skin with feathers, were found on the flank or in the coelom of normal hosts of 17 days of incubation. The Creeper factor in double representation restricts the developmental potencies, but it has shown no cell-lethal or organ-lethal effect in the structures which have been transplanted so far. Similar potency tests applied to primordia which have not yet been investigated should indicate whether the immediate cause of the lethality resides in a single organ or if death is caused by deficiencies in an integrating mechanism.

EARLY DETERMINATION AND CAUSATION OF THE CREEPER CHARACTERS

Previous experiments have shown that the limb primordia of normal 48-hour chick embryos will differentiate into normal wings and legs if transplanted heterotopically.

(Certain qualifications of this statement with respect to the pigment cells, which must be supplied by the host, with respect to details of joint formation, which depend on complete innervaton, and with respect to size reduction do not concern us in the present discussion.) The present experiments show that the modification of development which is caused by the Creeper factor is likewise fixed by at least as early as the fifty-fourth hour of incubation and cannot be reversed by implantation into a genetically normal host. This holds for the Cpcp as well as for the CpCp primordia. At the time of operation the latter are thickenings or small buds. The fact that the skeleton differentiating from early lethal CpCp embryos is identical with that of phocomelic embryos is further convincing evidence that both are genotypically identical. All the morphological, histological, and growth abnormalities are faithfully reproduced in Cpcp transplants. As was to be expected the development of most transplants was impaired, in addition to the Cp effect, by the transplantation per se, partly in the form of an accentuation of the Creeper characteristics and partly in the form of hypodactyly, hypophalangy, and other deficiencies which are not among the Creeper symptoms but are characteristic of normal limb transplants.

It must be emphasized that the relative self-differentiation of the Creeper phenotype is demonstrated only for the particular conditions of the present experiments. It is possible that primordia removed at earlier stages or reared in a genetic milieu different from White Leghorn might behave differently.

The fact that the transplants were incorporated in the normal host circulation deserves special emphasis. The limb primordia were transplanted in a developmental stage at which their blood vessels were not differentiated yet. Therefore, they were supplied with normal nutritive material and normal hormones throughout their differentiation. It results that all agents carried in the blood circulation are ruled out definitely as causal factors in chondrodystrophy and phocomelia. Landauer (1929) and Fugo and Witschi (1938) have come to the same conclusion with respect to hormone deficiencies. The former author has failed to find appreciable abnormalities in the most important hormone-secreting glands of chondrodystrophic chicks, and the latter investigators have found that hypophysectomy at 33–38 hours of incubation does not result in chondrodystrophy.

NONSPECIFIC GROWTH RETARDATION AS THE PRIMARY GENE EFFECT

Landauer has shown that the structural deficiencies of Creeper embryos, as they develop, are accompanied, or even preceded, by growth reductions. He interprets the primary gene effect as a general growth retardation of the entire embryo at early stages of development, the localized phenotypic manifestations merely reflecting a differential susceptibility of the primordia at the stages when the growth-restricting agents are in operation. The morphogenetic and histogenetic abnormalities of the Creeper skeleton are considered as secondary effects. Such an interpretation implies a causal relation between growth and histogenesis. Chondrodystrophy and phocomelia are considered as expressions of differing degrees of an early growth restriction, according to the single or double representation of the Creeper factor.

If the Creeper factor is a growth inhibitor of a general, nonspecific nature, then it should be possible to simulate its effect by exposing normal limb buds to unfavorable, growth-restricting conditions. The *in vitro* experiments of Fell and Landauer (1935) were based on this reasoning. These authors reared normal leg buds in an ordinary and in a special "growth-restricting" medium. The abnormalities which they found re-

sembled the phocomelic condition in so many structural details that the authors were inclined to assume both to be "due to agencies of the same nature" (p. 149). Similarly, David (1936) found that cartilages developing from normal leg buds on the chorio-allantoic membrane lack occasionally (but not always) the cartilage zones near the epiphyses, which deficiency, again, is characteristic of phocomelia.

By the same reasoning one would expect that a less severe growth restriction imposed on normal limb buds might result in a phenocopy of the Cpcp (Creeper) condition, likewise that a low-grade growth restriction superimposed on the Cpcp condition might result in deficiencies intermediate between chondrodystrophy and phocomelia, or perhaps in pure phocomelia. Neither one of these expectations was fulfilled in our experiments. The heterotopic transplantation of normal limb buds leads to a size reduction which averages about 20 per cent (Hamburger and Waugh, 1940) and thus provides a suitable test. This growth restriction is ascribed to an insufficient vascularization, innervation, etc., and is therefore of a general, nonspecific nature. Many of these transplants show no morphogenetic abnormalities or histogenetic deficiencies whatsoever. Others exhibit abnormalities, such as hypodactyly, etc., which are typically different from chondrodystrophy. The only deficiency which both have in common is a size reduction of the tibia (and fibula) in excess of that of other elements. This, however, is rather an argument against the thesis to be tested, since the excessive growth reduction is not accompanied by a "chondrodystrophic" bending of the tibia or a thickening, etc., of the fibula. Typical Creeper features were found in only 1 transplant among more than 100; this case was interpreted as originating from an incidentally chondrodystrophic donor (a nonhereditary form of chondrodystrophy occurs sporadically in chicks [Dunn, 1927]).

Likewise, the Cpcp transplants did not give a graded series of transitional stages between chondrodystrophy and phocomelia. It is true that the smallest Cpcp transplants had the external appearance of phocomelic limbs; but, after the skeleton had been stained with methylene blue, a clear distinction between Cpcp transplants and phocomelic limbs was possible. An unfailing criterion is the typical hourglass shape of the phalanges and the clear segregation and articulation of metacarpals, metatarsals, and phalanges in Cpcp transplants (Fig. 2) in contrast to the fusion of these elements in phocomelic wings and legs (Figs. 5 and 7). Likewise, the erosion of the diaphyseal cartilage and the periosteal ossification are characteristic of the majority of Cpcp transplants but completely missing in CpCp skeletons. These differences are maintained despite an overlap in size.

These findings seem to indicate that transplantation as such is not an adequate method of producing phenocopies of the Creeper type. It is, of course, possible that other experimental procedures will result in such phenocopies. However, the fact that not every nonspecific growth restriction imposed on an early limb bud will produce automatically the Creeper symptoms seems to us of significance for the interpretation of the action of the Creeper factor.

Our data do not favor the assumption that the morphogenetic and histogenetic effects of the Creeper factor are secondary results of a primary growth restriction, as is implied in Landauer's hypothesis. They suggest an alternative explanation of its action, i.e., morphogenetic, histogenetic, and growth disturbances might be derived from a more basic deficiency. We agree with Landauer in his emphasis on the universal nature of the action of the Creeper gene and are inclined, therefore, to assume that we are dealing with some deficiency in a general metabolic or respiratory mechanism. This deficiency might concern a specific substance, or interfere with a specific reaction involved in such a general

activity. The localized manifestations would be due to differential susceptibility of the various primordia for such a deficiency. This assumption would explain why the histogenesis of one type of tissue—chondrogenesis, for example—is impaired more seriously than the differentiation of other tissues, such as the musculature which seems to proceed normally even under the CpCp condition. Growth, morphogenesis, and histogenesis might be affected independently of one another and even at different stages of development. There is no difficulty in accommodating the experimental data of Fell and Landauer (1935) to such a scheme. The medium used in their tissue-culture experiments might have precluded the elaboration of a specific agent which is required for chondrogenesis. This effect might be quite independent of its growth-restricting effect.

While we find ourselves in agreement with Landauer's most recent discussion of the action of the Creeper factor (1941) in which he likewise considers specific deficiencies (in the elaboration of enzymes, etc.) operative in all parts of the embryo and throughout development, we cannot follow him in his emphasis on growth disturbances as their primary effects. However, it is evident that our present data are strongly suggestive of, but give no crucial evidence for, an independence of histogenetic and of growth disturbances.

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PLATE I

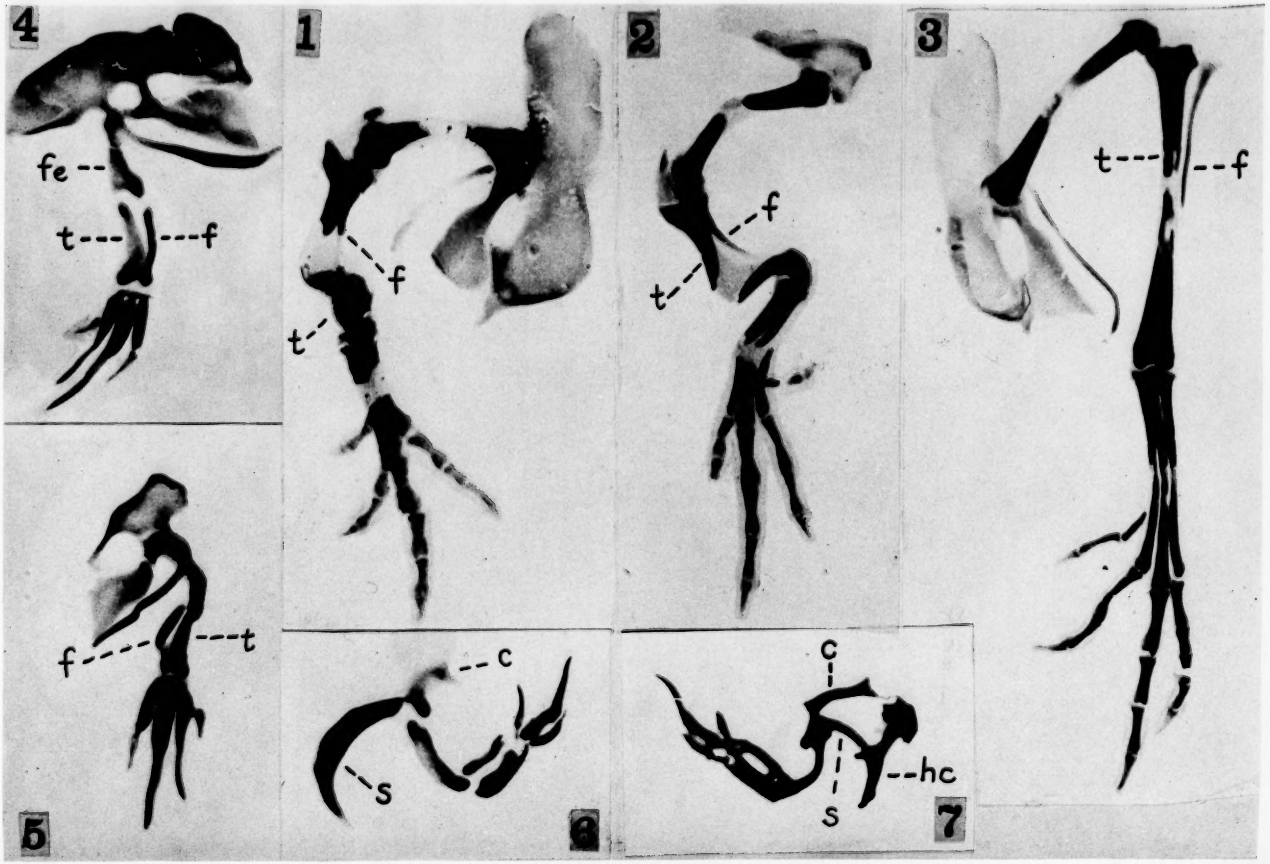

PLATE I

FIG. 1.—Cl 84. Left leg and pelvic girdle of chondrodystrophic (Cpcp) donor embryo. Fixed at 11 days of incubation. Methylene blue; ×6.3; *f* = fibula; *t* = tibia.

FIG. 2.—Cl 84. Right leg of the same embryo, transplanted in Stage 4 (small bud; 67 hours of incubation) to the flank of a normal embryo of the same stage. Fixed at 11 days of incubation. Methylene blue; ×6.3; *f* = fibula; *t* = tibia.

FIG. 3.—Cl 84. Control. Right leg of host of transplant in Fig. 2. Fixed at 11 days of incubation. Methylene blue; ×5.3; *f* = fibula; *t* = tibia.

FIG. 4.—Left leg of a phocomelic (CpCp) embryo, for comparison with Fig. 5. Fixed at 12 days of incubation. Methylene blue; ×5; *fe* = femur; *f* = fibula; *t* = tibia.

FIG. 5.—Cl 51. Right leg of an early lethal (CpCp) embryo transplanted in Stage 3 (very small bud; 64 hours of incubation) to the flank of a normal embryo of the same stage. Fixed at 12 days of incubation. Methylene blue; ×5.5; *f* = fibula; *t* = tibia.

FIG. 6.—Right wing of a phocomelic (CpCp) embryo, for comparison with Fig. 7. Fixed at 12 days of incubation. Methylene blue; ×5; *c* = coracoid; *s* = scapula.

FIG. 7.—Cl 96. Right wing of an early lethal (CpCp) embryo transplanted in Stage 1 (thickened somatopleure; 27 somites; 54 hours of incubation) into coelomic cavity of normal embryo, Stage 3 (very small wing buds; 68 hours of incubation). Transplant developed on inside of body wall, near right host wing. Fixed at 17 days of incubation. Methylene blue; ×3; *c* = coracoid; *hc* = host coracoid; *s* = scapula.

EFFECT OF THYMUS GLAND, THYMUS EXTRACTS, AND THYMUS DERIVATIVES ON GROWTH AND METAMORPHOSIS OF AMPHIBIAN LARVAE

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ALTHOUGH many years have elapsed since experiments were first performed on the thymus gland, the precise physiological role of this organ still has not been definitely established. Considerable interest in the problem has been rearoused recently by the results obtained with Hanson's thymus extract by Rowntree and his associates (1936*a*, *b*), and with thymocrescin—another type of thymus extract—by Asher (1936) on the development of the rat. The major portion of the work has been conducted on mammals, and for a description of the literature in this field the reader is referred to the reviews by Anderson (1932), Gudernatsch (1937), and Nelson (1939). A considerable amount, however, has also been performed on amphibians. This is not surprising since, in such material, growth and differentiation processes may be favorably studied in large numbers of genetically similar animals in the comparatively short time of the metamorphic period. As in mammals, very few noncontroversial findings have emerged. Gudernatsch (1912, 1917) and Romeis (1914) reported retardation of metamorphosis and increased growth in developing larvae which were fed thymus substance. Since these effects were obviously opposite to those produced by thyroid material, a thymus-thyroid antagonism was postulated. On the other hand, Swingle (1917), Uhlenhuth (1919), Romeis (1925), Abderhalden (1926), and Janes and Segaloff (1938), employing either feeding or immersion techniques, indicated that the thymus gland plays no significant role in amphibian development.

Criticism, however, may be lodged against the methods employed by the foregoing investigators. The failure by some to obtain positive results with fed thymus material may possibly be due to a destruction of the active thymic principle, if it does exist, in the alimentary tract. The immersion method, likewise, may be criticized, since its use necessarily implies that the principles can be absorbed through the integument. In view of the recent success obtained with injections of thymus extracts in mammals, it was considered worth while to subject the problem in amphibia to an extensive reinvestigation using the more precise injection technique.

MATERIALS AND METHODS

Two series of experiments were performed, one on *Rana pipiens* and the other on *R. palustris*. *R. pipiens* eggs were secured from several healthy female frogs and fertilized according to the methods described by Rugh (1934). *R. palustris* eggs were obtained from a running stream in the open field.

In the first series of experiments approximately 800 *R. pipiens* tadpoles were used. They were immersed in tap water which was changed daily and kept at a temperature of 19°–22° C. Crowding effects were minimized by placing sets of 25 tadpoles into sepa-

rate 2-liter glass bowls. The animals were divided into eight groups of approximately 100 animals each. Injections were begun at the hind limb bud stage.

Group I received injections of 0.05 cc. of a suspension containing the equivalent of one to two adult *R. pipiens* thymi ground in frog Ringer's solution. These glands were dissected from approximatley 2,000 frogs during the course of the experiment. They were usually injected within 1–2 hours after dissection. On certain occasions they were stored before being used for no longer than 24–48 hours in the freezing compartment of a refrigerator. The animals of Group II were injected with 1–2 mg. of thymocrescin[1] dissolved in 0.05 cc. Ringer's. Each milligram corresponded to 0.45–0.65 gm. of fresh calf thymus. Group III was given injections of 0.05 cc. of Hanson's thymus extract.[1]

In view of the fact that Schaffer *et al.* (1938) have recently claimed that Hanson's extract is rich in iodine-reducing compounds such as glutathione and cysteine, we thought it advisable to test these materials also. Consequently, the animals of Groups IV and V were injected, respectively, with 0.25–0.50 mg. glutathione (Hoffman–La Roche) and 0.25–0.50 mg. cysteine hydrochloride dissolved in 0.05 cc. Ringer's.

Groups VI, VII, and VIII served as controls. Group VI received injections of 0.05 cc. of a suspension containing 1–2 mg. of fresh frog skeletal muscle ground in Ringer's. Group VII comprised the untreated controls. Another untreated group (VIII) was employed to control the effects of Group III since unavoidably the Hanson's extract injections were begun approximately 3 weeks later than the others. All injections, in this first series, were administered twice a week for a period of 12 weeks.

In the second series approximately 450 *R. palustris* tadpoles were used. These were segregated into four groups and kept under conditions similar to those of the first series. Injections were again initiated at the hind limb bud stage. Group A received injections of 1–2 mg. thymocrescin in 0.05 cc. Ringer's, Group B, 0.05 cc. Hanson's extract, and Group C, 1–2 mg. crystalline egg albumin in 0.05 cc. Ringer's. Group D consisted of untreated controls. In this series each experimental animal received a total of 20 injections over a period of 8 weeks.

All materials, with the exception of Hanson's extract, were freshly prepared immediately before each injection. No metal instruments were used in handling the glutathione and cysteine. All injections were made intraperitoneally through a narrow glass capillary tube in the younger animals and through a 27-gauge hypodermic needle later in the experiment. After being injected, each animal was placed, for a few seconds, on its dorsal side on a piece of moistened absorbent cotton. In test animals, with the use of colored dyes, it was found that with this method very little, if any, of the injected material was lost upon reimmersing the animals.

In preliminary trial tests several of the substances employed, particularly Hanson's extract and cysteine hydrochloride, were found to be extremely toxic. This was due largely to the high acidity of these substances and so, in both series of experiments, all materials were brought to pH 6.5 immediately before injection. No precipitation occurred with any of the substances at this pH. With this procedure, only a very small number of deaths occurred in all the groups throughout the course of the experiment.

All animals were fed a diet consisting largely of boiled spinach, occasionally supplemented with boiled liver and rolled oats during the experimental period. It was con-

[1] We are greatly indebted to Mr. Albert Segaloff and Dr. Warren O. Nelson of the department of anatomy, Wayne University College of Medicine, for fresh thymocrescin made by Asher's method, and for Hanson's extract.

sidered likely that, if the various thymus substances, extracts, and derivatives employed exerted any truly endocrine effects, these should be revealed even though the animals were fed a normal diet.

At certain intervals after initiation of treatment, the total (from snout to tip of tail), trunk (from snout to base of tail), and hind limb lengths of each animal were measured as accurately as possible to the nearest millimeter. The animals of each group were then collected in a net, and, after the excess water had been allowed to drip through the net, the total weights were determined, from which the average weight of the animals in the various groups could be computed. It is realized that weights determined in this way represent only an approximation. The nature of the experiment made it impracticable to utilize the dry-weight method. In any event, as will be seen below, the wet-weight data are in accord with other results on the same animals.

TABLE 1

EFFECT OF THYMUS MATERIALS ON DEVELOPMENT OF *Rana pipiens* LARVAE

Group No.	Treatment	Date	*TL*	*TrL*	*HL*	Percentage *M*	Weight (gm.)
I.....	Frog thymus	March 19	70.3±1.39	24.8±0.39	10.1±0.72	0	3.2
		April 21	79.3 2.93	26.8 0.59	31.2 2.34	41.3	5.2
II.....	Thymocrescin	March 19	72.7 0.97	25.8 0.37	10.5 0.56	0	3.5
		April 21	79.9 1.50	26.8 0.37	30.3 1.50	56.0	4.8
IV.....	Glutathione	March 19	70.6 1.17	24.7 0.39	8.7 0.46	0	3.1
		April 21	79.0 1.42	25.1 0.39	26.4 1.33	45.8	4.7
V.....	Cysteine	March 19	69.8 1.33	23.8 0.48	10.4 0.70	0	3.2
		April 21	77.3 1.63	25.5 0.60	31.8 2.15	49.0	4.6
VI.....	Muscle	March 19	64.8 1.32	22.8 0.44	7.8 0.64	0	2.6
		April 21	74.7 1.87	24.0 0.47	28.4 2.13	27.6	3.9
VII.....	Untreated	March 19	72.6 0.99	25.2 0.41	8.9 0.51	0	3.7
		April 21	80.0±2.43	26.9±0.59	27.8±1.78	50.0	5.0

TL, *TrL*, and *HL* represent the mean total, trunk, and hind limb lengths (±standard error) of those animals not as yet exhibiting extruded fore limbs after 16 (March 19) and 24 (April 21) injections.

Percentage *M* represents the percentage of animals showing both fore limbs extruded.

Weight (gm.) is the mean wet weight (in grams) of animals not as yet exhibiting both fore limbs extruded.

RESULTS

In Table 1 may be seen the data for the first series of experiments after 16 and 24 injections had been administered to *R. pipiens* tadpoles. After 16 injections no appreciable differences could be noted in the total, trunk, hind limb lengths, or weights of the tadpoles in any of the groups, with the exception of those in the muscle group which appeared to be somewhat retarded in both growth and metamorphosis, the latter being judged at this time by hind limb length. None of the animals in any of the groups had extruded fore limbs at this time. Even after 24 injections, the total, trunk, hind limb lengths, weights, and percentage of the animals which had metamorphosed had not been significantly altered, with the exception again of those animals which had received the muscle injections. These had been delayed in their growth and differentiation processes.

Table 2 gives the results for Hanson's extract on *R. pipiens* tadpoles together with a group of untreated controls. After 10 injections, no significant differences in the two groups could be determined. By the eighteenth injection, however, the animals treated with Hanson's extract were more advanced in metamorphosis, as judged by hind limb

TABLE 2

EFFECT OF HANSON'S EXTRACT ON DEVELOPMENT OF *Rana pipiens* LARVAE

Group No.	Treatment	Date	*TL*	*TrL*	*HL*	Percentage *M*	Weight (gm.)
III.....	Hanson's extract	March 19	68.6±0.80	25.0±0.30	8.1±0.34	0	3.0
		April 21	82.4 1.14	27.8 0.29	32.4 1.25	49.9	5.3
		May 12	.....	.....	.....	84.4	.....
VIII.....	Untreated	March 19	66.8 1.24	24.0 0.45	7.5 0.54	0	2.9
		April 21	79.4±1.50	25.2±0.48	24.6±1.71	33.3	4.3
		May 12	.....	.....	.....	63.3	.....

TL, *TrL*, and *HL* represent the mean total, trunk, and hind limb lengths (±standard error) of those animals not as yet exhibiting extruded fore limbs after 10 (March 19), 18 (April 21), and 24 (May 12) injections.

Percentage *M* represents the percentage of animals showing both fore limbs extruded.

Weight (gm.) is the mean wet weight (in grams) of animals not as yet exhibiting both fore limbs extruded.

TABLE 3

EFFECT OF THYMUS EXTRACTS ON DEVELOPMENT OF *Rana palustris* LARVAE

Group	Treatment	Date	*TL*	*TrL*	*HL*	Percentage *M*	Weight (gm.)
A.....	Thymocrescin	July 13	54.3±0.75	17.6±0.21	9.7±0.84	0	1.4
		August 3	61.8 0.89	19.7 0.24	17.4 1.32	43.0	2.0
B.....	Hanson's extract	July 13	56.9 0.72	18.4 0.18	11.0 0.75	0	1.5
		August 3	66.0 0.98	20.6 0.25	22.8 1.43	65.1	2.8
C.....	Albumin	July 13	52.3 0.77	17.0 0.22	7.1 0.70	0	1.2
		August 3	58.5 1.02	18.3 0.27	15.0 1.24	22.2	1.4
D.....	Untreated	July 13	55.0 0.89	17.6 0.22	9.0 0.82	0	1.4
		August 3	63.2±1.44	19.7±0.34	16.2±1.61	48.7	1.9

TL, *TrL*, and *HL* represent the mean total, trunk, and hind limb lengths (±standard error) of those animals not as yet exhibiting extruded fore limbs after 13 (July 13) and 20 (August 3) injections.

Percentage *M* represents the percentage of animals showing both fore limbs extruded.

Weight (gm.) is the mean wet weight (in grams) of animals not as yet exhibiting both fore limbs extruded.

length and percentage showing fore limbs, than were the untreated controls. The injected animals were also slightly larger in size and heavier. After 24 injections, 84.4 per cent of the treated animals had both fore limbs extruded, whereas only 63.3 per cent of the untreated controls had reached this stage of development.

In Table 3 are listed the results for the second series of experiments. The data corresponding to 7 injections of the various materials are not given since these showed that

no significant effects had as yet been obtained. After 13 injections, however, several slight differences between the various groups were noted. The tadpoles receiving the albumin injections were somewhat retarded in general body growth and degree of metamorphosis, whereas those to which Hanson's extract was administered were, on the whole, slightly more advanced in metamorphosis, although their general body size and weight were not significantly different from those of the untreated controls. Thymocrescin was without any effect at this time. After 20 injections, the differences between these groups were more marked. Crystalline egg-albumin injections had produced a definite delay in metamorphosis and an inhibition in growth. The animals treated with Hanson's extract were, as in the first series of experiments, slightly larger in size and heavier and definitely more advanced in metamorphosis as seen both from the number of animals which exhibited fore limbs and from the hind limb lengths of the remaining animals. The thymocrescin-injected animals showed measurements similar to those of the untreated controls.

DISCUSSION

It would appear from the above results that among the substances employed the only one capable of causing an increase in the growth and rate of metamorphosis of *Rana* tadpoles is Hanson's extract. The effect on growth is slight, whereas the influence on differentiation is more marked. Both processes are apparently affected to a greater extent in the later stages of metamorphosis. If the effects obtained in the present experiments with Hanson's extract are to be interpreted in terms of its sulphhydryl content (Rowntree *et al.*, 1938) it is difficult to see why such substances as glutathione and cysteine are without influence on the development of the metamorphosing anuran. Since, however, Rowntree *et al.* have claimed that the effect of such compounds is not so great as that of the whole Hanson's extract, one might assume that the sulphhydryl in the extract is present in some more potent form, perhaps linked with some other organic component, or that other substances present in the extract also contribute to the activity. There is also the possibility that, in our experiments, glutathione and cysteine were not administered in a dosage necessary to give rise to the effect.

The effect of the Hanson's extract may, on the other hand, be purely a nutritional one. The diet which was administered to all the animals throughout the experiment was quite adequate in that the untreated controls, as a whole, metamorphosed without any apparent abnormalities. All animals in this group, both in the early and in the later stages of metamorphosis, were healthy and vigorous. The diet, however, may not have been an optimal one and may have been supplemented by the addition of the nutritive materials present in the extract.

The reason why Hanson's extract produces a relatively greater effect in the later stages of metamorphosis is also difficult to understand. If it acts synergistically with the thyroid as some have claimed (Colosi, 1933) or antagonistically toward it as others have reported, one would expect some effect to be exerted in the earlier stages also. Moreover, as is well known, thyroid substance causes a more rapid metamorphosis at the expense of growth, whereas Hanson's extract, as was seen, causes an acceleration of both processes.

It is to be emphasized that the effects obtained with Hanson's extract, although significant, are slight in degree and hardly endocrine-like in nature. If it were possible to

rear and inject amphibians through successive generations as Rowntree and his associates have done with rats, the effects of the extract might have been augmented.

The results also indicate quite clearly that no significant effects were obtained with thymocrescin or ground frog-thymus material. It is interesting to note that, although thymocrescin contains labile sulphur, recent analyses by Segaloff (quoted by Nelson, 1939) have shown it to be devoid of compounds with the sulphhydryl linkage, and this may account for its inability to affect the development of anurans. Our original purpose in employing frog thymus was based largely on analogy with the methods used by several investigators who showed that injections of homozoic pituitary substance are more effective than that of other species in inducing developmental processes in the amphibia (Rugh, 1935). No such endocrine influence, however, was obtained in our experiments with frog thymus. The possibility still remains that some effect on development might have occurred if extracts of the glands, prepared by the methods of Hanson, had been employed.

It was believed at first that the crude nature of the protein in the injected frog-muscle material was the cause for its observed inhibitory effect on tadpole development. As can be seen from our results, however, injections of crystalline egg albumin—a purified protein—produced the same effect. We are, at the present, unable to explain this inhibitory influence of frog muscle and crystalline egg albumin on the development of anuran larvae.

SUMMARY

1. Intraperitoneal injections of Hanson's thymus extract produced a significant, although slight, increase in the growth and differentiation of *R. pipiens* and *R. palustris* larvae reared on a normal diet.

2. Injections of adult *R. pipiens* thymi, glutathione, and cysteine hydrochloride were without effect on the development of *R. pipiens* larvae.

3. Thymocrescin failed to exert any significant effect in both species of *Rana*.

4. In *R. pipiens*, frog-muscle injections were found to cause a delay in growth and differentiation. This same effect was obtained in *R. palustris* with crystalline egg albumin.

5. On the basis of these results, it is possible to conclude that thymus substance, extracts, or derivatives do not exert any obvious hormonic influence on growth and differentiation of developing anuran larvae.

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INHIBITION OF LACTATION IN THE RAT[1]

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LITERATURE on the endocrine regulation of the mammary gland has been reviewed by Nelson (1936) and Turner (1939). It suffices to mention here that mammary growth is dependent upon ovarian secretions, which exert their characteristic effect only if the anterior pituitary is intact. Once structurally developed, the mammae secrete milk when stimulated by an anterior lobe lactogenic hormone. This latter hormone in turn exerts its characteristic function only when a concurrent adrenotropic-adrenal cortical activity is present (reviewed by Gaunt, 1937).

Still unsettled is the problem as to how lactogenic hormone secretion, and hence milk secretion, is regulated. In some forms at least nervous influences are involved (Selye, 1934; Ingelbrecht, 1935; Herold, 1939), and certainly endocrine factors are concerned.

Nelson, working primarily on the guinea pig, developed the theory that the secretion of estrogen by the ovary and placenta was a factor normally holding lactation in abeyance during pregnancy. This was thought to be accomplished by a twofold action: first, a direct action on the mammary glands and, second, a prevention of lactogenic hormone secretion. Folley and Kon (1938) suggested that any substance which stimulates mammary growth inhibits lactation. Many have found in various species (references by Folley and Kon, 1938) an inhibition of lactation by estrogens. The dosage needed, however, probably varies greatly with the species.

The presence or absence of an ovary, in the rat at least, modifies the suppressing action of estrogen on lactation, but opinions differ as to the extent of this ovarian influence (Anselmino and Hoffman, 1936; Folley and Kon). De Jongh's (1933) experiments indicated that the well-known inhibiting effects of gonadotropins on lactation might not be entirely through their stimulation of ovarian activity, since chorionic gonadotropin stopped lactation in ovariectomized as well as in normal mice. This suggests the converse of the mechanism described by Bates, Riddle, and Lahr (1937) in which lactogenic hormone suppressed production of gonadotropin in birds.

We report here a further investigation of endocrine factors which inhibit lactation and of conditions modifying their action in rats. An abstract of this work has been published (Edelmann and Gaunt, 1940).

METHODS

The amount of lactation of the experimental rats was judged by the growth rate of their litters, reduced to 4 young each. Hormone injections were begun on the day of delivery if not otherwise stated. Ovariectomy was performed on the day of delivery. Injections were generally carried out for 15 days, the age at which the young of normal

[1] This work was aided by a grant from the American Philosophical Society. It was started at the Biological Laboratory, Cold Spring Harbor, during the summer of 1938. We are grateful for the assistance of Mr. Samuel Joseph.

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litters began to eat. Controls consisted of 15 normal untreated mothers with litters reduced either to 4 or to 6 members. Such differences in litter size did not cause results to vary. One death occurred in the control group of 80 suckling young.

RESULTS[2]

Chorionic gonadotropin (*Table 1, Ser. 1*).—The pregnancy urine hormone stimulates mammary development in intact rats; and ovariectomy in such stimulated animals is followed by a spontaneous milk secretion (Selye, Collip, and Thomson, 1933). It has been reported (Cannon, 1938) and denied (Hathaway, Davis, Reece, and Bartlett, 1939) that this hormone by itself has a marked inhibitory effect on established lactation in rats, although the latter authors found it to augment the lactation-inhibiting action of estrogen (Reece *et al.*, 1940). In our hands it was without appreciable effect in 6 intact rats in doses of 100–500 RU daily if given for not more than 5 days. With a 15-day injection period there was some inhibition in two cases. This may or may not have been a specific action of the hormone since Hathaway *et al.* found such extracts somewhat toxic in large doses.

We were more interested in the report of De Jongh that APL would inhibit lactation in castrate as well as in normal mice. With castrate rats, given doses of 100 RU per day for 15 days, an inhibition of lactation was found in only one of four cases, and this in view of the more extensive negative results with pregnant mare serum was probably not significant. None of the young died.

Pregnant mare serum (*Table 1, Sers. 2, 3, 4*).—PMS in doses of 0.5–1.0 cc. per day was the most effective inhibitor of lactation we have used. It gave a relatively quick response and resulted in the death of many young. In castrate animals, however, it was entirely without effect, indicating that a high level of gonadotropin is not per se detrimental to the release of lactogenic hormone. The inhibitory effect in intact animals was more marked if treatment was started 5 days before delivery.

Anterior pituitary extract (*Table 1, Sers. 5, 6*).—Gaunt and Tobin (1936) found in lactating adrenalectomized rats given cortical extract that lactation was diminished by the injection of anterior pituitary extract (Squibb). It was suggested that this inhibition was due to the estrin-producing properties of gonadotropins in the extract, despite the fact that the latter were slight in amount. When tested here in normal animals, intact or castrate, it had no effect on lactation. Its effect on adrenalectomized animals must have been in some way dependent upon the peculiar condition of those animals.

Estrogenic hormone (*Table 1, Sers. 7–13*).—Estrone (Amniotin) in doses of 200 IU per day inhibited lactation in intact animals but was without effect in castrates in doses of 300–5,000 IU per day. This finding is in apparent agreement with Anselmino and Hoffman (1936).

Using larger doses of estrogen (1 mg. per day of either estradiol-17-propionate or stilboestrol), we obtained an inhibition of lactation such as was obtained by Folley and Kon in castrate animals, but distinctly less than that obtained with similar doses in in-

[2] The endocrine products used here were generously supplied us as follows: chorionic gonadotropin (Follutein), Anterior Pituitary Extract, Amniotin and stilboestrol by Dr. J. A. Morrell, E. R. Squibb & Sons; pregnant mare serum (Gonadin Serum) by Mr. Donald Wonder, Cutter Laboratories; testosterone propionate (Oreton) and progesterone (Proluton) by Dr. Erwin Schwenk, Schering Corporation; and estradiol-17-propionate (Follacro) by Dr. E. W. Blanchard, Schieffelin & Co.

TABLE 1

MEAN FIGURES ON THE GROWTH RATES OF LITTERS OF RATS AFTER VARIOUS TREATMENTS OF THE MOTHER

Treatment and Condition of Mother	No. Cases	On Fifth Day		On Ninth Day		On Thirteenth Day		On Sixteenth Day		Weight* Change in Mothers (Gm.)
		Weight Surviving Young (Gm.)	Percentage Dead	Weight Surviving Young (Gm.)	Percentage Dead	Weight Surviving Young (Gm.)	Percentage Dead	Weight Surviving Young (Gm.)	Percentage Dead	
1. Chorionic gonadotropin, 100 RU/day, castrate	4	9.1	0.0	13.3	0.0	17.8	0.0	20.0	0.0	− 6.8
2. Pregnant mare serum, 0.5–1.0 cc./day, intact	7	10.3	0.0	11.5	7.2	12.4	46.4	14.1	71.4	+11.0
3. Pregnant mare serum, 1 cc./day, castrate	4	10.7	0.0	17.3	0.0	23.0	0.0	.....	0.0	+18.0
4. Pregnant mare serum, 0.5 cc./day, intact, pretreated†	2	5.3	50.0	6.0	75.0	.....	100.0	.....	.....	.....
5. AP extract, 1–2 cc./day, intact	7	10.9	0.0	16.1	0.0	22.5	0.0	28.0	0.0	+25.0
6. AP extract, 1–2 cc./day, castrate	4	11.3	0.0	19.1	0.0	25.6	0.0	30.0	0.0	+ 6.3
7. Amniotin, 200 IU/day, intact	5	11.8	0.0	15.1	0.0	15.9	25.0	20.7	45.0	+ 9.3
8. Amniotin, 300–5,000‡ IU/day, castrate	8	13.4	0.0	19.7	0.0	24.6	0.0	.....	0.0	− 8.0
9. Amniotin, 200 IU/day, intact, pretreated§	4	9.6	0.0	10.0	25.0	11.1	56.3	.....	.....	0.0
10. Estradiol-17-propionate, 1 mg./day, intact	3	9.7	0.0	9.0	16.6	12.8	50.0	16.5	66.6	−10.3
11. Estradiol-17-propionate, 1 mg./day, castrate	5	10.7	0.0	13.5	0.0	16.3	0.0	18.5	0.0	−14.2
12. Stilboestrol, 1 mg./day, intact	6	9.7	0.0	10.1	0.0	10.7	45.0	15.2	75.0	−16.3
13. Stilboestrol, 1 mg./day, castrate	7	9.3	0.0	11.9	4.1	15.0	25.0	17.1	33.3	−12.5
14. Testosterone propionate, 3 mg./day, intact	5	7.5	0.0	10.8	50.0	11.5	56.2	13.3	75.0	+ 7.0
15. Testosterone propionate, 3 mg./day, castrate	4	9.7	0.0	13.4	8.3	15.8	8.3	16.9	8.3	+14.0
16. 1 mg. stilboestrol+3 mg. testosterone propionate, castrate	1	4.0	0.0	5.0	0.0	.....	100.0	.....	.....	+12.0
1 mg. stilboestrol+3 mg. testosterone propionate, castrate.e	1	10.0	0.0	15.7	0.0	17.3	0.0	20.5	0.0	0.0
17. 1 mg. stilboestrol + 2 mg. progesterone, castrate	1	10.2	0.0	11.5	0.0	12.0	0.0	13.0	25.0	0.0
1 mg. stilboestrol+2 mg. progesterone, castrate	1	10.0	0.0	9.5	0.0	.....	100.0	.....	.....	0.0
Controls, untreated	15	9.0	0.0	14.5	0.0	20.1	1.2	25.0	1.2	+ 5.0

* Weight change during injection period only.

† Treatment started 5 days before delivery.

‡ One received 300, 2 received 500, 1 received 1,000, 1 received 200, and 3 received 5,000 IU per day. No difference was found for the different dosages.

§ Treatment started 4 days before delivery.

tact mothers. If estrogen administration was started 4 days before delivery, the lactation-inhibiting response was considerably augmented.

Testosterone propionate (*Table 1, Sers. 14 and 15*).—Male hormone in doses of 3 mg. per day inhibited lactation markedly in intact mothers, as found by Folley and Kon (1938) and by Robson (1937), but like the estrogens it was much less effective in castrates.

Hormone combinations (*Table 1, Sers. 16 and 17*).—In two cases 1 mg. of stilboestrol was combined with 3 mg. testosterone propionate as a daily dose, and in two cases a similar amount of stilboestrol was given together with 2 mg. progesterone. Since the individual members of each pair responded differently, the cases are tabulated separately. There was, however, probably a greater inhibition than would have been expected from stilboestrol alone in such cases. Opportunity for performing conclusive experiments on this point with progesterone, the more significant substance, has not been available.

Weight change in mothers.—The effects of the various substances injected on the body weight of the mothers are probably of some significance as indicated below. Because of considerable variability, however, they must be interpreted cautiously. The great majority of our normal rats gained a little weight during the first 15 days of their lactation period. With large doses of estrogen a gain of weight never occurred and definite weight losses were the rule. Such results are entirely compatible with the well-known weight-reducing effect of these substances in nonlactating animals (references by McEuen, Selye, and Collip, 1937; and Folley and Kon, 1938). Testosterone-treated animals, on the other hand, never lost weight and in nearly every case gained—again as expected from the experiments of the above workers. Combinations of either testosterone or progesterone with stilboestrol seemed to cancel out the weight-reducing action of the latter, as Nelson (1938) and Korenchevsky, Hall, and Burbank (1939) found under other circumstances. Weight gains with anterior pituitary extract were to be expected since the preparation was rich in growth hormone. With pregnant mare serum there was in no case a weight loss and, on the average, weight gains as great as or greater than those in untreated animals. This indicates that if any sizable amount of estrogen was released as a result of this treatment it was counteracted by a simultaneous production of other steroids.

DISCUSSION

It is clear that the lactation-inhibiting properties of gonadotropins are mediated entirely through the ovary, but the absence of weight loss in mothers suggests that nonestrogenic steroids may be involved. Unless large doses are given, estrogen by itself—i.e., in castrate animals—is not a potent inhibitor of lactation in the rat. In intact animals it is much more effective, suggesting that under estrogenic stimulation the ovary releases additional substances which assist in the inhibition of lactation. A dose of estrogen can be found which decreases lactation only in intact animals, thus explaining the results of Anselmino and Hoffman, who detected no effects in castrates. The greater inhibition in intact animals is probably associated with the well-known luteinizing action of estrogen on rat ovaries (Hohlweg, 1934). The additional material secreted by such ovaries might be progesterone, a substance which, however, by itself does not stop lactation in doses of 1 mg. per day (Folley and Kon). It could, however, under physiological conditions act synergistically with estrogen or act in some way to prevent lactation initiation without the ability to inhibit an established milk secretion as suggested by Ansel-

mino, Herold, Hoffman, and Pencharz (1936). Ovarian androgens could possibly be involved, since they are released in detectable amounts in various experimental conditions (Hill, 1937; Papanicaloau and Falk, 1938; Greene and Burrill, 1939).

An alternative explanation has been suggested to us (by Dr. Hans O. Haterius) to explain the greater effectiveness of estrogen in the intact as compared to the castrate animal. In the intact rat the liver, engaged in the inactivation of endogenous estrogen, may be less able to handle the large additional amounts given here and hence is more responsive than the castrate.

Results and conclusions given here must be limited to rats. In other species, such as the guinea pig, it takes less estrogen to suppress lactation, and there is a shorter latent period before any effects of treatment started at delivery can be observed (Nelson, 1934). The large doses of estrogen demanded by the rat for the inhibition of lactation and the long latent period for any effect raises question as to its physiological importance during gestation in this species (see also Turner, 1939). If the ovary has such a physiological role in the rat, it may be partially by means of some steroid other than estrogen, and we prefer such an interpretation of results found here.

SUMMARY

1. Chorionic gonadotropin did not inhibit lactation in castrate rats.

2. Pregnant mare serum inhibited lactation markedly in intact rats, but not at all in castrates.

3. An anterior pituitary extract that inhibited lactation in adrenalectomized rats did not do so in normal animals.

4. Estrogens in doses of 1 mg. per day inhibited lactation in both intact and castrate rats, but much less so in the latter. A dose could be found which would inhibit lactation in intact animals only.

5. Testosterone propionate acted like the estrogens and was a more effective lactation inhibitor in intact than in castrate animals.

6. In a limited series combinations of estrogen and progesterone or testosterone did not give decisive results.

7. Indirect evidence is given that when the ovary is stimulated by gonadotropin or estrogen it releases nonestrogenic substances which assist in the inhibition of lactation. The physiological importance of any direct action of estrogen in inhibiting lactation in the rat is questioned.

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EXPERIMENTAL MODIFICATION OF BREEDING CYCLES IN GOATS[1]

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RECENT studies on the modification of sexual or breeding cycles of field mice, ferrets, raccoons, guinea pigs, ground squirrels, cottontail rabbits, and other mammals with limited annual breeding periods confined to the seasons of long or lengthening days, shows that some, but not all of them, are sexually photoperiodic. Within limits, the breeding cycles of the sexually photoperiodic ones can be modified at will by altering experimentally the duration of their daily periods of exposure to light (Bissonnette, 1936, 1938; Marshall, 1936, 1937; Wells and Zalesky, 1940; and others cited by them).

Some animals, however, breed not in the long-day seasons but in those of short or shortening day. Brook trout, sheep, deer, and goats are some of these. The change-over of the breeding seasons of the three last-named species when they are transferred to the Southern Hemisphere (Marshall, 1937), and the success of Hoover and Hubbard (1937) in causing a December-spawning strain of brook trout to spawn and produce viable, well-growing, healthy offspring in August, suggested that similar success might be obtained with goats. Such control and shifting of breeding cycles will make possible the "freshening" of some members of large flocks throughout the year. It would enable breeders to bridge the hiatus which normally occurs from late August to late January, because goats have a gestation period of 21–22 weeks and fail to breed between early April and September.

The hope of success led to the experiment to be described here, planned and carried out jointly at the Hillshires Farm, Killingly, Connecticut, with the aid and supervision of Emil Kaiser, chief herdsman and technician there.

MATERIALS AND METHODS

Four female Toggenburg goats and one female Nubian goat were housed in a small room off the main stable for females, in which about one hundred and forty others served as controls under exactly similar conditions of feeding, care, and ventilation. Both groups were turned out to pasture by day when the weather was suitable. Daily periods of pasturing conformed to the schedules of lighting of the two groups.

One Toggenburg male was confined in a separate stable about 100 yards from the stables for females, under feeding and lighting conditions similar to those of experimental females.

Controls were subject to natural conditions of lighting throughout the experiment except for being brought into the well-lighted stable at the usual hours. These hours varied somewhat with the seasons and weather and were the same as those for the experimental animals except as stated below.

Experimental animals received normal daylight throughout the early part of the experiment with light added at night from a 100-watt bulb, placed in front and a little

[1] Aided by grants from the Penrose Fund of the American Philosophical Society, 1938–40, and by goats and their care and keep from Hillshires Farm, Killingly, Connecticut.

above them in such position that they could not hide their eyes from its direct rays when it was lighted. During later parts of the experiment they were subject to reduction of daily periods of daylight below normal for the season by being driven into their stable at times specified and the room darkened by light-tight shutters over its windows. This, of course, made their nights during this part of the schedule periods of total darkness unaffected by moonlight or evening or morning twilights. The electric lights in the two experimental stables were wired on the same circuit controlled by an electric time switch. This

TABLE 1

TIME SCHEDULE FOR EXPERIMENTAL GOATS

Date of Change of Time	Time Switch On	Added Hours of Light (+) or Hours Less (−)	Time Switch Off (S) or Blinds Closed (B)
Jan. 25	5.05	+1	6.05, S
Feb. 4	5.17	+2	7.17, S
14	5.30	+3	8.30, S
24	5.42	+4	9.42, S
Mar. 6	5.54	+5	10.54, S
16	6.06	+6	12.06, S
26	6.18	+7	1.18, S
Apr. 5	6.29	+6	12.29, S
12	6.37	+5	11.37, S
19	6.44	+4	10.44, S
26	6.52	+3	9.52, S
May 3	7.00	+2	9.00, S
10	7.08	+1	8.08, S
17	7.15	0	.....
24	8.22	−1	7.22, B
31	8.28	−2	6.28, B
June 7	8.33	−3	5.33, B
14	8.37	−4	4.37, B
21	8.40	−5	3.40, B
28	8.40	−6	3.15, B to 6.00 A.M.
July 5	8.40	−6	3.15, B to 6.00 A.M.
6	All on normal schedule and out in pasture day and night		

P.M. E.S.T. (Time Switch On: Jan. 25 – Mar. 26)

A.M. (Time Switch Off: Mar. 26)

P.M. E.S.T. (Time Switch Off: Jan. 25 – Mar. 6)

P.M. (Time Switch Off: Apr. 5 – May 10)

P.M. D.S.T. (Time Switch On: May 24 – July 5)

P.M. D.S.T. (Time Switch Off: May 24 – July 5)

was set to give the schedule of added electric light for the days following, up to May 24 (Table 1). From that date onward the animals were driven into the stable before sunset and the blinds closed to reduce the daily period of light below normal (Table 1), on a schedule reducing it 1 hour each week to 6 hours on June 28. This period was then adhered to until July 5, when the experimental animals were all turned out to pasture and left day and night with the male. During the period of reduced lighting, the blinds were opened during the night before morning twilight each night, so that reductions were made only at one end of the day until June 28, after which blinds were opened at 6:00 A.M. After July 5, lighting was normal for the season.

The females used were numbered 38T, 39T, 40N, 49T, and 52T, the "T" and "N" signifying Toggenburg and Nubian, respectively. All remained in good health throughout the months (over a year) during which they were under observation.

OBSERVATIONS AND RESULTS

Controls showed latest heat periods on or before March 15 and no normal ones thereafter until after September 1. Occasional very short and weak signs—almost, if not quite, unnoticeable and too short for breeding unless the animals were running continuously with the male—occurred, but they were of too short duration to last until the female could be taken to the male. Such heat periods are seldom, if ever, effective in producing offspring.

TABLE 2

HEAT PERIODS OF EXPERIMENTAL FEMALES BETWEEN FEBRUARY AND JULY 5*

Animal	First Heat	Second Heat	Tried with ♂	Reaction	Remarks
38T	.....	.....	.....	.....	No heat
39T	May 17	June 14	June 14	Mated	♂ not aggressive
40N	May 29	June 16, 17	June 16, 17	Not mated	♂ not interested
49T	.....	.....	.....	.....	No heat
52T	May 28	.....	.....	.....	No heat

* None of the heat reactions were normal, as they occurred in autumn. The male mated but once with one female and was not interested later.

TABLE 3

MATINGS AND RESULTS SUBSEQUENT TO JULY 5 AS SHOWN BY BIRTHS IN DECEMBER

Animal	Mating Date	Delivery	Sex of Kids	Milk Record
38T	July 15	Dec. 15	2♂♂	Good milker
39T	.....	.....	.....	No milk
40N	July 14	Dec. 14	1♂+1♀	Light milker
49T	July 16	Dec. 16	1♂	Good milker
52T	July 11	Dec. 11	1♂+1♀	Light milker

Experimentals were not noticed in heat after some dates in February until 39T showed the twitching of the tail and receptivity that characterize heat, on May 17. Two others showed signs of heat in May, and two of these three females again showed heat signs in June, at which times they were tried with the experimental male and one was served, or mated (Table 2). None of the heat reactions occurring in May and June with these females was normal in intensity or completeness, and the male was not very aggressive in his attempts to mate.

After being turned out to continuous pasture with the male, four of the females must have mated at least once and probably more times, for in December they gave birth to kids, as shown in Table 3 along with the calculated probable dates of effective matings and the number and sex of the kids born.

DISCUSSION AND CONCLUSIONS

It is doubtful that the sudden increase of daily lighting following July 5 was the cause of the heat periods of the four females giving birth to kids in December. That question will receive attention in subsequent experiments. It seems much more probable that the continued reduction of daily lighting was becoming increasingly effective in inducing oestrous cycles and heat periods, and the short-time shift to longer days in the pasture failed to inhibit the next heat period in line by reducing the animals' gonadotropic activity below the threshold for induction of heat. It is also possible that a slight shift in the proportions of the hormones concerned may have occurred to bring on such a quick and uniform mating response in the 6 days which appear to have covered the fertile matings of the four reacting animals out of the five used.

In any case, the results of the experiment indicate that reversal of the seasonal cycle of length of daily period of exposure to light of both Toggenburg and Nubian goats leads to partial reversal of the sexual or breeding cycles of the animals. Artificial increase of daily lighting was followed by cessation of heat periods in February instead of in March for experimental animals. Reduced lighting, with temperatures not varying from the normal for the seasons, was accompanied or followed by initiation of heat periods in May and June instead of in September in experimental animals. Even though the signs of heat were neither so intense nor so prolonged as usual, in these experimental animals in May and June, and led to only one mating and no kids, the fact of their appearance is evidence that altered lighting is effective in controlling the oestrous cycles of these animals. The heat periods in July, following return to normal daylight cycles, cannot be open to the above objections, for they led to four pregnancies and production of seven kids.

It is probable that further study may make possible a schedule of controlled lighting which will induce fertile matings at desired intervals in the anoestrous or sterile period of the female goat. In any case the hiatus of births and the reduced milk production consequent to it can be bridged in by this method of controlled lighting. The births in December are almost 2 months before the normal time of first spring births.

SUMMARY

1. Increasing daily light periods from January 25 to April 5—followed by diminishing periods until July 5, while temperatures remained normal for the seasons, with four Toggenburg female goats and one male Toggenburg and one Nubian female—led to cessation of breeding cycles in February instead of March, followed by initiation of breeding cycles in May and June instead of September. Controls retained normal breeding cycles.

2. Matings in July within 10 days of return to normal lighting and continuous pasture with the experimental male produced seven kids from four of the five females used, all born between December 11 and 16, respectively. This is in contrast with first births from controls in February.

3. Two of the four females producing kids were good milkers under these conditions; the other two were light milkers.

4. Results indicate that breeding cycles in goats are controlled by daily periods of light in such a way that short days induce breeding cycles while long days inhibit them. The temperature cycle is not a major factor in environmental control of sexual or breeding cycles in goats.

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MAXIMAL DENSITY AND PHASES OF DEATH IN POPULATIONS OF GLAUCOMA PIRIFORMIS

(Six figures)

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THE growth of protozoan populations in pure cultures has not been investigated extensively, although the observations of Phelps (1935, 1936) and Loefer (1936) have indicated that growth of ciliates is not markedly different from that of bacteria. In fact, Phelps (1935) has described several of Buchanan's phases of growth—initial stationary, lag, logarithmic growth, negative growth acceleration, and maximal stationary phase—in cultures of *Glaucoma piriformis*. However, the observations of Phelps were limited to the first few days of incubation, and complete histories of populations are known for only one ciliate—*Paramecium bursaria* (Loefer, 1936). Hence, present knowledge of the later phases in growth of protozoan populations is very limited. In view of this situation, the writers have attempted to trace growth of *G. piriformis*, with particular attention to the phases following the maximal stationary phase in which maximal density of population is reached.

MATERIAL AND METHODS

The bacteria-free stock strain of *G. piriformis* and the experimental cultures were grown in the following solution: casein-peptone (Difco Tryptone), 10.0 gm.; KH_2PO_4, 1.0 gm.; distilled water, 1.0 liter. No adjustment of pH was necessary, and the initial pH in all series was 6.3 ($\pm$0.1). The medium was tubed in 9.0-cc. amounts for each series and then sterilized in the autoclave for 20 minutes at 122° C. Stock cultures for inoculation of the various series were grown in flasks of this medium.

Two different preparations of casein-peptone (Tryptone) were used. The two samples differed in date of purchase, in color, and in the populations which they supported. The older preparation was relatively light in color and supported maximal densities of approximately 150,000 ciliates per cubic centimeter. The medium containing this peptone is referred to below as Medium A and was used in Series I–III. The second sample, purchased about a year later than the other, was darker in color and supported much lower populations, as described in Series IV, V, and IX. The medium containing the darker peptone is referred to as Medium B. In Series VI–VIII, the two types of peptone were mixed, as specified below.

Each tube in the experimental series received a 0.5-cc. inoculum from a young stock culture. After inoculation one tube was used for determination of initial pH, and several were fixed for the initial counts (number of ciliates per cubic centimeter). During incubation several tubes were fixed at each of the intervals indicated in the separate series; the counts from these tubes, after correction for evaporation, trace growth of the various populations. Our method of counting has been described previously (Hall, Johnson, and Loefer, 1935).

All series were incubated in darkness. Series I was maintained in an incubator at 24° C. for 13 days; at this point, failure of a power line necessitated transfer to room temperature (observed range, 24°–26°). Series III was incubated at room temperature at a range of 22°–26°. The remaining series were incubated at 25° (±0.1).

GROWTH IN TWO DIFFERENT MEDIA

Series I.—This series, inoculated from a 42-hour stock culture, showed an initial count of 5,425 ciliates per cubic centimeter. The maximal stationary phase, in which the population approximated 154,000 per cubic centimeter, was reached during the fourth day and was maintained for less than 48 hours (Fig. 1). Thereafter, the population showed

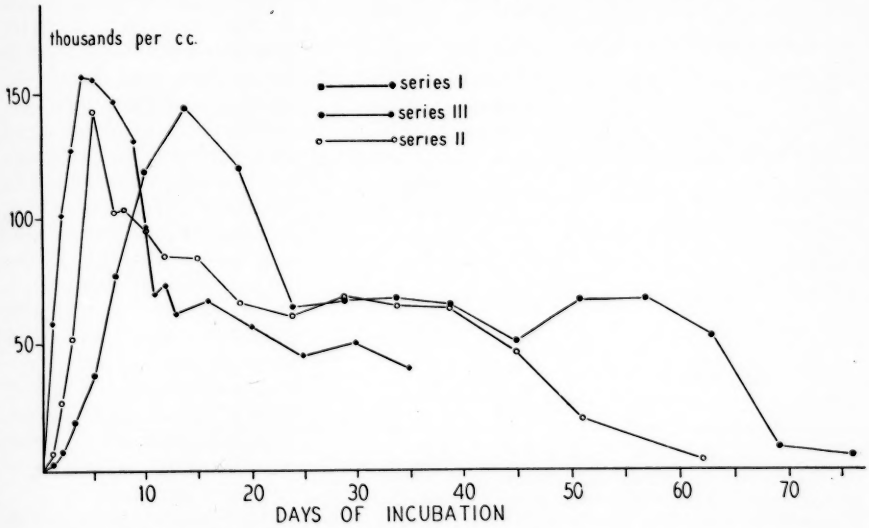

FIG. 1.—Growth of populations in Series I–III, decribed in point-to-point curves. Counts were discontinued at the last point indicated in each series. However, *G. piriformis* continued to live for 2–7 months longer, as noted in the text.

a rapid decline to approximately 60,000 per cubic centimeter on the tenth day. During the next 25 days there was a gradual decline to approximately 40,000 ciliates per cubic centimeter. At this point only a few tube cultures remained, and these were saved for subsequent observations and viability tests. Active ciliates were still present in the cultures at the end of 8 months.

Series II.—A 22-hour stock culture was used in inoculating this series, and the initial count was 490. Growth was traced for approximately 2 months (Fig. 1). The maximal density of population (maximal stationary phase) was reached about the fifth day of incubation. By the seventh day a rapid decrease in population was well under way, and this decline continued, although at a decreasing rate, for more than 2 weeks. This phase was followed by a stationary phase of approximately the same duration, in which the population remained practically constant. After about 40 days of incubation, another

phase of death was initiated, and the population was reduced to less than 25,000 per cubic centimeter by the fifty-first day. Subsequently, the rate of death decreased, although there was a further drop in density to approximately 3,000 per cubic centemeter at the end of 62 days. Residual cultures still contained active ciliates at the end of 4 months, when observations were discontinued.

Series III.—In this series, inoculated from a 20-hour stock culture, the initial count was 180. The maximal density of population (Fig. 1) was reached later than in either Series I or Series II, and the subsequent decline in population was somewhat less rapid. The secondary stationary phase following the first phase of death lasted approximately 30 days and was succeeded by a second phase of death similar to that observed in Series II. Counting was discontinued after 76 days, and the few remaining cultures were preserved for later observations. Active ciliates were still present in small numbers after 8.5 months of incubation.

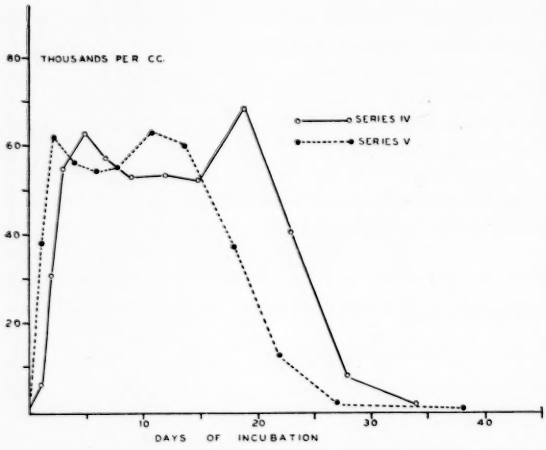

FIG. 2.—Growth of *G. piriformis* in Series IV and V (Medium B). Counts were discontinued at the last point in each series, although residual cultures still contained living ciliates at the end of 3.5 months.

Series IV and V.—In these two series Medium B was substituted for Medium A. Otherwise the experimental procedure was unchanged. Series IV was inoculated from a 28-hour culture, and Series V from a 51-hour stock culture. The respective initial counts were 800 and 3,125 ciliates per cubic centimeter.

In Series IV (Fig. 2) the population had increased to a density of approximately 63,000 per cubic centimeter on the fifth day of incubation. Thereafter, the density remained within the range 53,000–68,000 per cubic centimeter for approximately 2 weeks. This phase was followed by a major phase of death, during which the population dropped to about 8,000 per cubic centimeter within a period of 9 days. During the next 7 days, however, the decline was less rapid; furthermore, cultures incubated for later examination still showed active ciliates after 3.5 months.

Growth in Series V (Fig. 2) was similar to that in Series IV, although the phase of maximal density appeared earlier and passed earlier than in the preceding series. Just as in Series IV, residual cultures still showed active ciliates at the end of 3.5 months.

The observations on Series I–III suggest that the normal growth curve of *G. piriformis* in Medium A approximates that described in Figure 3. Within the first 2 weeks of incubation the population reaches a maximal stationary phase (or phase of maximal density), which is maintained for not more than 2–4 days. This phase, in which the population approximates 150,000 per cubic centimeter, is followed by a phase of death lasting for a week or more. Subsequently, there is a secondary stationary phase in which

the population remains almost constant or else declines gradually over a period of 2–4 weeks. A second phase of death follows, and this is succeeded by a tertiary stationary phase in which a slowly decreasing population may be maintained at a low level for several months.

The type of growth curve in Medium B (Fig. 3) differs in several respects from that characteristic of *G. piriformis* in Medium A. The phase of maximal density in Series IV and V shows a population of less than half the density observed in Series I–III and in this respect is comparable to the secondary stationary phase mentioned above. After the peak is first reached in Series IV and V, the population remains fairly constant for 10

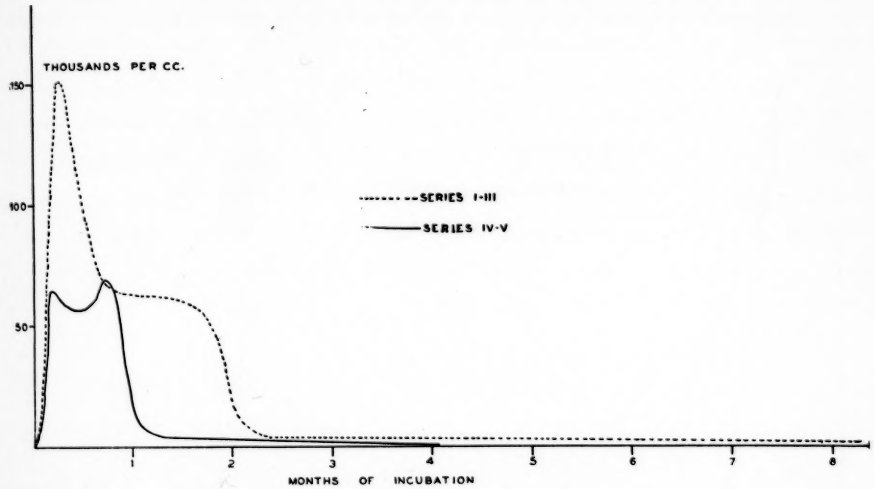

FIG. 3.—Generalized growth curves for *G. piriformis* in two different peptone media. The curve with the higher peak is characteristic of Medium A (Sers. I–III); that with the lower peak, of Medium B (Sers. IV and V).

days or more, variations falling within the range of 10–20 per cent. This phase of maximal density is succeeded by a phase of death, so that at the end of 30 days or more the population is reduced to the general level observed in Series II and III after approximately 2 months of incubation. Subsequently, the population may be maintained at a low level for at least an additional 2 or 3 months.

DIVISION RATES IN SERIES I–V

Calculated division rates (generations per day) for Series I–V afford no explanation for the differences in the growth curves characteristic of the two media. On the first day of incubation (Fig. 4) the number of generations ranged from 2.9 to 3.6, but series in both media are represented at the two extremes. After the first day the decrease in division rate was rapid in every case. Of the five series, I and V are closely comparable with respect to generation times, although the two series were maintained in different media. Likewise, Series II, III, and IV are somewhat similar for the first 3 days of in-

cubation; Medium B was used in Series IV and Medium A in the other series. A comparison of initial counts shows that Series I (5,425 ciliates per cubic centimeter) and Series V (3,125) are fairly comparable; Series II (490), Series III (180), and Series IV (800) may be grouped together on the same basis. The generation curve for Series III, with an initial density of 180 ciliates per cubic centimeter, shows a more gradual negative slope than that of any other series. It thus appears that the division rate for the first 2 or 3 days is correlated with the initial density of population rather than with the type of peptone used in the medium.

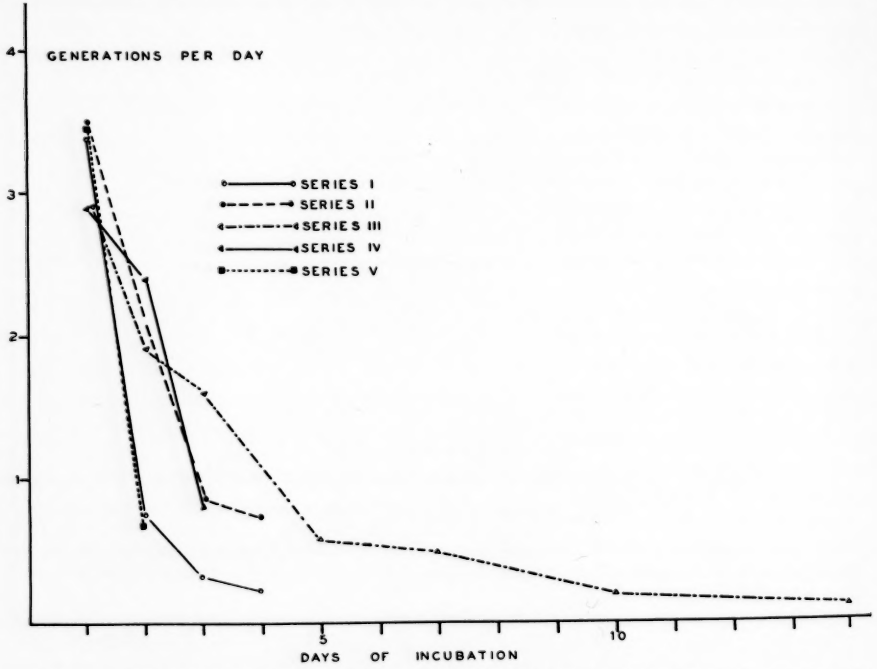

FIG. 4.—Number of generations per day in Series I–V. The final point in each curve coincides with that at which maximal density of population is attained.

GROWTH IN MIXTURES OF TWO PEPTONES

The striking differences between the populations supported by the two peptone media suggested the following observations on mixtures of the two peptones.

In Series VI our two samples of casein-peptone were mixed in the proportion of four parts light peptone to five parts dark peptone. Each tube was inoculated from a 22-hour stock culture in Medium B, and the initial count was 116 per cubic centimeter. The resulting growth curve (Fig. 5) is intermediate between the type observed in Series I–III and that described for Series IV and V, and the maximal density (112,000 ciliates per cubic centimeter) is proportionately intermediate. Active ciliates were still present in residual cultures after 3 months.

Series VII was similar to Series VI, except that the solution of light peptone (Medium A) had been stored in a flask for approximately 7 weeks after sterilization. This was mixed with a fresh solution of dark peptone (Medium B) in the same proportion as in Series VI. The series was inoculated from a 28-hour stock culture in Medium B, and the initial count was 800 per cubic centimeter. Correlated with the higher initial density, there was a sharper rise to the phase of maximal density than was observed in Series VI, and the major phase of death was initiated at least a week earlier. Residual cultures contained living ciliates at the end of 3.5 months. Like Series VI, Series VII presents a growth curve of the intermediate type and thus demonstrates that, whatever the factor responsible for a high maximal density may be, this factor is still operative after sterile Medium A has been stored for several weeks.

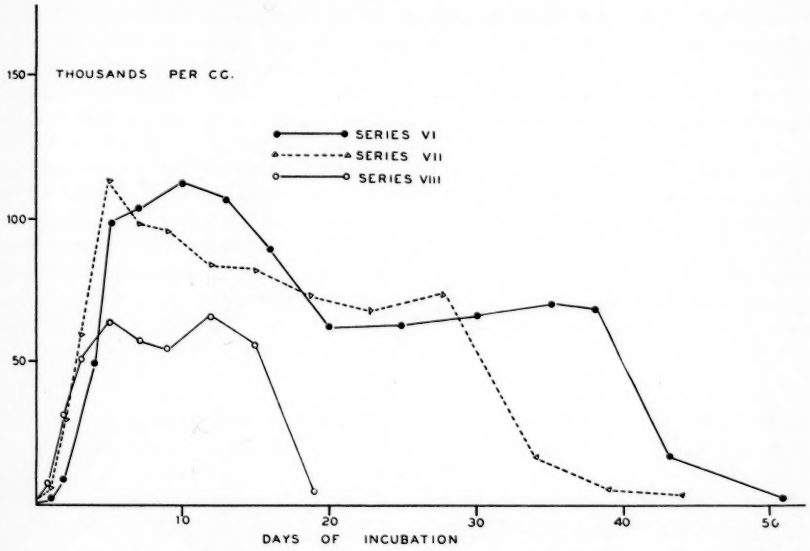

FIG. 5.—Growth in mixtures of the two peptones, Series VI–VIII

Series VIII represents an attempt to determine whether or not the factor favoring dense populations is still detectable in material from old cultures of *G. piriformis* in Medium A. The medium contained four parts of fresh Medium B and five parts of a filtrate from a 50-day culture in Medium A. The series was started from a 28-hour stock culture in Medium B, and the initial count was 800. For the first few days of incubation, growth of the population (Fig. 5) was similar to that in Series VII, in which the initial density was also 800 per cubic centimeter. In Series VIII, however, the population reached a peak of approximately 60,000 per cubic centimeter, just as in Series IV and V—a density little more than half as great as that observed in Series VI and VII. Thus the effect of adding the culture filtrate was the same as if no light peptone at all had been added to the medium.

EFFECTS OF ADDED THIAMINE (VITAMIN B_1)

The results obtained in Series I–VIII suggested that one of our preparations of casein-peptone contains a factor which, in appropriate concentrations, supports a large population for a short period and maintains a moderately dense population for a month or more thereafter. When the concentration of this substance is reduced, as in a mixture of the two peptones, the early rise in population is decreased proportionately (Fig. 5) and the

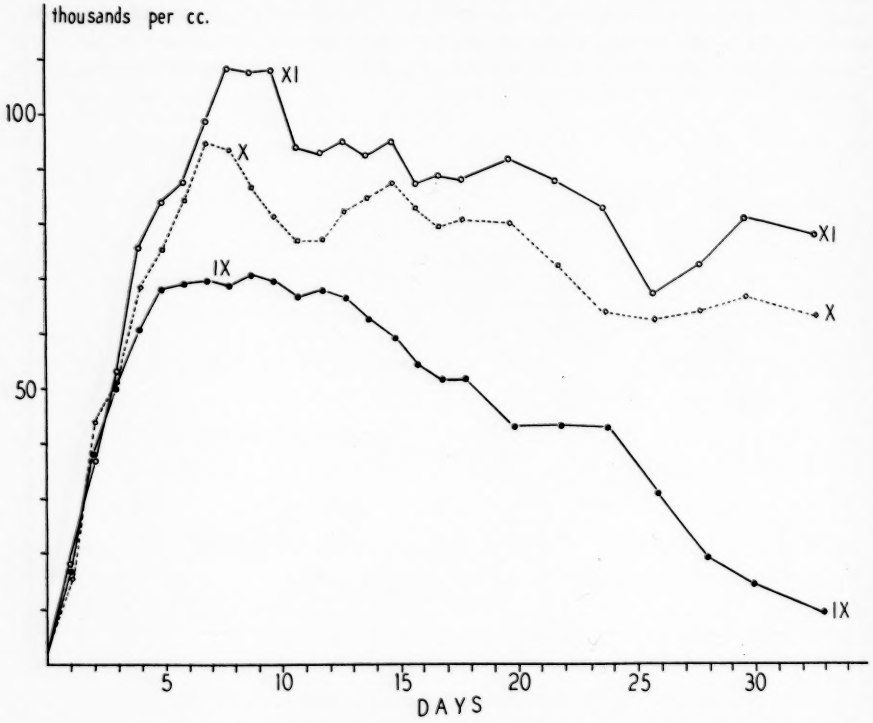

FIG. 6.—Growth of *G. piriformis* in Medium B with and without added thiamine. Series IX, control, Medium B; Series X, Medium B with thiamine, 1×10^{-7} gm. per cubic centimeter; Series XI, Medium B with thiamine, 1×10^{-6} gm. per cubic centimeter.

secondary stationary phase is shortened. This substance apparently does not accelerate fission; instead, its effect is to prolong the period during which the population continued to increase, and this effect is proportional to the concentration of the unknown substance. Whatever the factor may be, it apparently is destroyed or inactivated by the ciliates, since it is either absent or reduced to an ineffective concentration in old cultures (Ser. VIII). The apparent properties of this substance suggested that it might be a growth factor present in the casein from which our peptones have been derived. Since thiamine (vitamin B_1) is known to be essential to growth of several ciliates, it seemed

possible that our Medium B might contain less than an optimal concentration of this growth factor. Hence, this substance was added to Medium B in Series X and XI, as described below.

Series IX–XI.—Medium B was used as the control medium in Series IX; in Series X thiamine was added to this medium in a concentration of 1×10^{-7} gm. per cubic centimeter, while ten times as much thiamine was added in Series XI. All three series were inoculated from a 6-day culture of *G. piriformis* in Medium B, and the initial count was 1,832 ciliates per cubic centimeter. The three populations were traced for a period of 33 days.

In the controls (Ser. IX) the phase of maximal density was reached on the fifth day (Fig. 6), with a population of about 70,000 ciliates per cubic centimeter. With added thiamine (1×10^{-7} gm. per cubic centimeter) in Series X, the population approached 95,000 on the seventh day of incubation. In Series XI, with a higher concentration of thiamine (1×10^{-6}), the population reached a density of approximately 110,000 per cubic centimeter on the eighth day. In Series IX the population remained practically constant for about a week and then began a fairly steady decline. In Series X the population ranged between 78,000 and 88,000 per cubic centimeter during the second and third weeks of incubation and then dropped to a density of approximately 65,000 which was maintained for the last 10 days of incubation. In Series XI the population dropped slightly after the tenth day and then varied between 88,000 and 95,000 per cubic centimeter through the third week; during the last 10 days of incubation the number of ciliates ranged from 70,000 to 80,000, approximately.

The results demonstrate that the available concentration of thiamine definitely affects the density of population during the maximal stationary phase and the secondary stationary phase and also influences the time at which the major phase of death begins.

DISCUSSION

The present observations represent the first attempts to trace the later history of populations in cultures of *G. piriformis*. The early phases of growth in this species have been described by Phelps (1935) and hence have not been considered in detail. Our findings demonstrate phases not previously described for protozoan populations, and they also raise certain questions which cannot yet be answered completely.

As described for bacteria and yeasts, the maximal stationary phase merges into a "phase of accelerated death," and the latter leads into a phase characterized by a more or less constant death rate. In *G. piriformis*, on the other hand, the phases of death are more complicated. In one of our media the phase of maximal density is followed by a phase of death in which the population is reduced by more than one-half. This rapid decline is succeeded by a secondary stationary phase, in which the population may show a gradual decline or may remain practically constant for several weeks. This second stationary phase is followed by a major phase of death, in which the population drops to a low level within a period of approximately 1 week. Finally, the population enters a phase suggesting a tertiary stationary phase, in which the density decreases very slowly and is maintained at a low level for several months. The exact length of this final phase has not been determined, although living ciliates were still present in cultures of different series when observations were discontinued after 4.0–8.5 months of incubation.

The behavior of populations in a second preparation of casein-peptone was quite dif-

ferent, although the division rate of young cultures was apparently the same as for cultures in the other medium. Growth of the population in this second medium ceases at a density approximating 60,000–70,000 ciliates per cubic centimeter, and the population shows little change in density for the next week or two. Next there occurs a major phase of death in which the population is reduced to a low level. These phases are completed in about a month—approximately half the time required in the other medium. The final phase, however, is much the same in the two cases, since populations of low density have already persisted for over 3 months in the second peptone medium.

The results obtained in Series I–VIII indicate that one of our peptones (Medium A) contains a factor which does not accelerate fission appreciably but does increase the length of the period during which growth of a ciliate population continues. The substance may be present also in the second type of peptone (Medium B), but only in a concentration which supports a population of moderate density for a relatively short time. The effect exerted by the substance is proportional to its concentration, and the substance itself apparently is inactivated or destroyed by the ciliates.

A preliminary attempt to identify this postulated substance as thiamine is described in Series IX–XI. The results indicate that an adequate concentration of thiamine is essential to the secondary stationary phase observed in Series I–III. Series IX, in Medium B, showed a fairly steady decline in density of population after the second week of incubation; in Series X and XI, on the other hand, the behavior of the populations resembled that in the secondary stationary phase of Series I–III. In fact, the density of population in Series XI was appreciably higher than that in Series I–III during this phase. Hence it may be assumed that the concentration of thiamine used in Series XI was higher than that available in Medium A (Sers. I–III).

Such an assumption implies that thiamine cannot be the only factor controlling the phase of maximal density (approximately 150,000 ciliates per cubic centimeter) in Series I–III. Series X and XI show an early peak (Fig. 6) which is suggestive of the maximal stationary phase observed in Medium A, but the maximal density of population is in each case much lower than that in Series I–III. It seems probable that the effect of higher concentrations of thiamine would be limited, as far as the maximal stationary phase is concerned, since a tenfold increase in thiamine (Ser. XI) raised the population level less than 20 per cent above that observed in Series X. Theoretically, it would be necessary on this basis to increase the concentration of thiamine to the order of 1×10^{-4} gm. per cubic centimeter in order to produce a maximal stationary phase approaching that observed in Series I–III. Since such a concentration of thiamine presumably would exert proportional effects during the secondary stationary phase, the results would not duplicate the growth curves obtained in Series I–III. Hence it seems necessary to conclude that other factors as well as thiamine are probably involved in the appearance of the maximal stationary phase characteristic of Medium A (Sers. I–III).

Other possibilities are now being considered. Preliminary observations (Hall, 1942) indicate that riboflavin (vitamin B_2) is probably essential to growth of *Colpidium campylum* and suggest that nicotinamide may also be an essential growth factor for this ciliate. It is possible that these substances may be equally important in the metabolism of *G. piriformis*. Hence both riboflavin and nicotinamide are being tested for their effects on density of population in cultures of *G. piriformis*. These studies are being combined with observations on the effects of thiamine in concentrations higher than those used in Series X and XI.

In conclusion, however, it may be pointed out that the density of population in cultures of *G. piriformis* is related quantitatively to the concentration of thiamine and that the phases of death also are influenced markedly by the available concentration of this vitamin. Thus, Series X and XI showed populations of approximately 65,000 and 80,000, respectively, after 1 month of incubation, whereas Series IX (Medium B without added thiamine) showed a population of less than 10,000 ciliates per cubic centimeter. In other words, addition of thiamine to Medium B converted the major phase of death, as observed during the third and fourth weeks of incubation (Sers. IV, V, and IX), into a phase resembling the secondary stationary phase noted in Medium A (Sers. I–III).

These results have some bearing on the relation of protozoan waste products to the behavior of populations. The steady decline in Series IX after the second week of incubation, considered separately, might have been attributed to the accumulation of toxic wastes. However, the addition of thiamine in Series X and XI definitely postponed this major phase of death, although the populations reached higher levels than in Series IX and more waste products should have been produced within a shorter period. In unpublished observations on *C. campylum* a solution of thiamine was added to cultures after the maximal stationary phase was reached, while controls received the same quantity of sterile distilled water. The effect of thiamine added at this time was much the same as that observed in Series XI, in which the thiamine was added before inoculation of the cultures. Accordingly, it seems obvious that death of the majority of ciliates in Medium B (Sers. IV, V, and IX) within the first 4 weeks of incubation is dependent upon a deficiency of thiamine.

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